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TAIL MELANOPHORES OF XENOPUS IN NORMAL DEVELOPMENT AND REGENERATION [1]

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A peculiar group of light-sensitive melanophores occurs in the ventral fin of Xenopus larvae. Bles (1905) noticed that these chromatophores are contracted during the day, but expand at night, darkening the tail markedly. More recently, Bagnara (1957) has shown that the melanophores of the tail fin are directly photosensitive and refers to this response as the "tail darkening reaction." His suggestion that a photochemical system is involved has been supported by Van der Lek, De Heer, Burgers and von Oordt (1958) who point out that the degree of melanophore response is a function of light intensity.

Because of the above observations and in view of the report that the numerical density of tail fin melanophores is only slightly influenced by the hypophysis (Bagnara, 1957), it seems likely that the presence or absence of light, not the pituitary, is the primary effector of tail melanophore response. The chromatotrophic hormone (CTH) of the hypophysis exerts some influence on these cells, however, as was shown by Thing (1952) who used the tail fin melanophores for assay of such hormone preparations.

It is curious that these melanophores are restricted to the distal half of the ventral fin (Bles, 1905; Bagnara, 1957). Apparently, the tip of the tail provides an environment favorable not only for the establishment of a light-sensitive mechanism, but also for melanogenesis. That the caudal portion of the ventral fin is generally chromogenic is suggested by our previous observation (Bagnara, 1957) that guanophores, which are never present in the tail of normal Xenopus larvae, develop in the distal portion of such tadpoles which have been deprived of their hypophyses.

The present study concerns the responses of tail fin melanophores of Xenopus to changes in illumination during the course of normal development and regeneration, and compares these melanophores with those in other areas of the larvae.

Materials and Methods

The larvae of *Xenopus laevis* used in this investigation were reared from eggs obtained from natural spawning of our adult stock which are kept in outdoor tanks.

[1] Aided by grants G-4048 and G-6231 from the National Science Foundation.

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In a few cases, however, ovulation was induced with chorionic gonadotrophic hormone. Throughout the larval period, powdered nettle was employed for feeding. Experiments were carried out in a temperature-controlled room at temperatures of 21° C. during the spring and summer and 24° C. during the fall and winter. Illumination was from north-facing windows.

During some of the experiments, larvae were subjected to darkness for periods of thirty minutes to several hours. Thirty minutes is approximately the minimal time interval for achievement of the tail darkening reaction (Bagnara, 1957; Van der Lek *et al.*, 1958). Dark treatment was carried out in a closed drawer in a darkened room. After appropriate intervals of exposure to darkness, the larvae were immersed immediately in 25% formalin. Such treatment insures rapid fixation and prevents melanophore contraction during the fixing process. The same method of fixation was employed in all experiments in which degree of expansion was to be observed. For long-term preservation, the larvae were removed to 10% formalin.

For the tail regeneration experiments, young Xenopus larvae of stages 51 through 54 (stages of Nieuwkoop and Faber, 1956) were used because older animals usually conclude their metamorphosis before tail regeneration is completed. Tails were cut off with iridectomy scissors while the larvae were hovering in a stationary position. The cuts were made just anterior to the pigmented area. In this way, enough tail remained to allow the larvae sufficient mobility for feeding and for respiration. Care was taken to use only tadpoles on which none of the pigmented area of the fin remained.

In order to evaluate the degree of melanophore response, the melanophore index (M. I.) of Hogben and Slome (1931) was employed. This index is general enough to permit analysis even of types of melanophores which differ slightly from one another in form.

Results

1. Normal development of the fin melanophores

Tail fin melanophores begin to appear at stage 47 (Fig. 1). They are first seen immediately adjacent to the somitic area at the very tip of the tail. The pigmented region gradually increases in size so that at stage 51 the distal third of the tail fin contains many melanophores. By stage 52 the pigmented area has increased to cover the entire caudal half of the tail. The anterior boundary of the pigmented region forms a clear line of separation from the proximal portion of the ventral fin which contains no melanophores at all.

In their early developmental stages, the young tail melanophores are elongated and sparsely branched. As differentiation proceeds, new branches are added radially so that the expanded melanophores appear stellate. During stages of expansion, melanophores of the tail fin differ from those of the head and trunk in that their processes are thin and much more heavily branched. As tail pigmentation proceeds during the early larval period, new melanophores are added at the edge and tip of the fin. Actually, there is a gradient in degree of differentiation; thus, under expanded conditions, melanophores immediately adjacent to the somites are fully developed and stellate while those at the edge are elongated (Fig. 2). Between these two areas exist partially differentiated melanophores

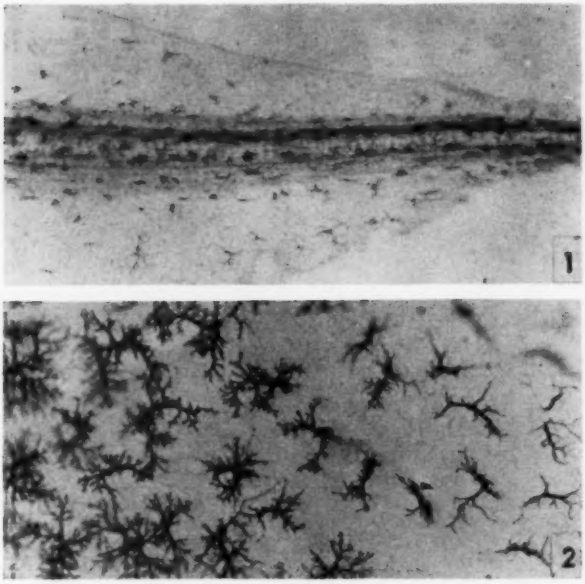

FIGURE 1. First appearance of tail fin melanophores at stage 47. Normal aquarium illumination. Magnification: × 10.

FIGURE 2. Ventral fin of stage 53 larva during the tail darkening reaction. Melanophore differentiation gradient from somites at left to fin edge at right can be seen in these expanded melanophores. Magnification: × 50.

with an intermediate number of branches. This suggests either that new melanophores are proliferating at the edge of the fin or that here unpigmented melanocytes are coming under the influence of a melanogenic substance which is distributed along a gradient from the somites to the ventral fin edge.

II. Normal development of the tail darkening reaction

Although not as striking as that of fully differentiated melanophores, the response of differentiating melanophores is discernible almost from their first appearance. Under normal room illumination, young melanophores are not able to assume the punctate form of the fully developed tail melanophores (Fig. 3); instead, they appear elongate with relatively few visible branches (Figs. 1 and 2). After exposure to darkness for thirty minutes or more, however, their branches become more obvious and often secondary branches can be seen (Fig. 2). Fully formed tail melanophores seen during the tail darkening reaction are stellate and possess secondary and tertiary branches (Fig. 4).

III. Regeneration of tail melanophores and the tail darkening reaction

Approximately four days after tail amputation, a new fin can be seen forming at the end of the regeneration blastema and at the cut edge of the ventral and

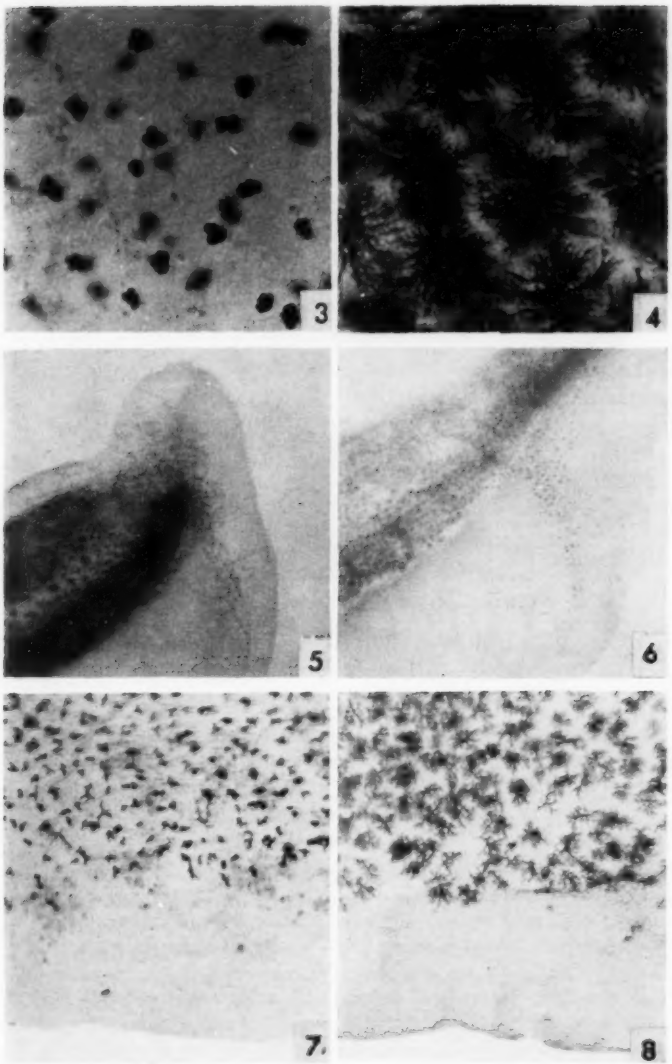

FIGURE 3. Mature melanophores of ventral fin in full contraction after bright illumination. Magnification: × 100.

FIGURE 4. Mature melanophores of ventral fin in full expansion during the tail darkening reaction. Magnification: × 100.

FIGURE 5. Fifth day of tail regeneration. Note melanophores streaming from the blastema at the line of amputation. Magnification: × 7.

FIGURE 6. Seventh day of tail regeneration. Melanophore streaming much more obvious. Magnification: × 7.

dorsal fins. At this time, the formation of melanophores begins in the new ventral fin near the base of the somitic blastema. During the next 6 or 7 days, these melanophores increase in number and appear as a stream of cells stemming from the regeneration blastema (Figs. 5 and 6). These melanophores are present only in the ventral fin and remain excluded from the area anterior to the original cut. Thus, the proximal area retains its unpigmented state. During the course of tail regeneration, tail melanophores gradually appear in the ventral fin, first making their appearance adjacent to the newly forming somites. After the tenth day of regeneration, the somitic core of the regenerating tail appears as a long spike to which is attached a generous ventral fin. Melanophores are abundant in the upper half of the fin near the developing somites, but are lacking in the lower half near the edge. After three weeks, tail regeneration is practically completed and the

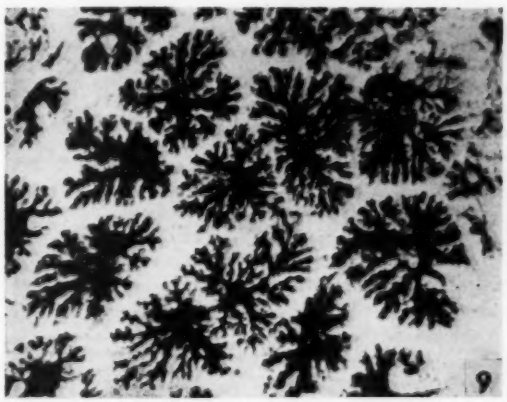

FIGURE 9. Melanophores typical of the head of mature larvae under normal illumination. Magnification: × 100.

new ventral fin is copiously supplied with melanophores. However, a clearly defined margin is present along the ventral edge of the fin (Figs. 7 and 8) which is permanently devoid of melanophores. Moreover, the melanophores at the edge of this margin are completely differentiated and there is no indication of any newly forming melanophores which might ultimately invade the clear margin. The unpigmented border extends along the complete length of the regenerated ventral fin except for the extreme proximal portion of the pigmented zone. This is the point at which the stream of melanophores was first seen in connection with the regeneration blastema. Apparently at the start of regeneration, a sufficient stimulus for melanization allows the complete reconstitution of pigmentation in this localized region. Presumably, in later stages of regeneration, the melanizing

FIGURE 7. Unpigmented area at edge of ventral fin at twenty-first day of regeneration. Slight tail darkening reaction is evident because larva was preserved immediately after removal from a murky aquarium. Magnification: × 10.

FIGURE 8. Larva similar to that in Figure 7. This larva, however, was placed in the dark before fixation; thus, the regenerated fin melanophores are fully expanded. Magnification: × 10.

stimulus is not sufficiently strong for complete repigmentation and thus the clear marginal area is formed.

Just as in normal development, the fully differentiated melanophores in the regenerating fin are light-sensitive (Figs. 7 and 8). Under normal illumination, the melanophores are punctate in appearance; but after exposures to darkness of thirty minutes or more, they expand and assume a stellate form. Differentiating melanophores in the tail regenerate are also light-sensitive just as they are during normal development (Fig. 2).

IV. Observations on other melanophores

In contrast to the melanophores of the tail fin which expand upon exposure to darkness, the large epidermal melanophores of the head (Fig. 9) contract after similar treatment. This reaction was observed empirically by Bles (1905), but no quantitative estimate of the response was recorded. In the present experiments, it was observed that the average M. I. for head melanophores of 64 tadpoles which were kept under normal illumination was 4.7, while the M. I. for 74 tadpoles fixed after exposure to darkness was 3.3. This response to changes in illumination is not as marked as that shown by the melanophores of the tail fin but it is clearly visible even macroscopically on the tadpoles which, except for the tail, appear to blanch after exposure to darkness.

Discussion

The fact that light-sensitive melanophores develop in the fin of Xenopus larvae poses several questions of embryological and physiological significance. First of all, what mechanism allows these peculiar light-sensitive melanophores to form only in the distal half of the fin, leaving the proximal portion completely free of this type of chromatophore? Stevens (1954) has shown that the posterior trunk tailfold areas of Xenopus neurulae are a good source of melanophores; thus the entire ventral fin has an adequate reservoir of melanoblasts upon which to draw. Realization of the melanophore potential is another matter, however, and may be dependent, as Twitty and Niu (1954) have suggested for urodeles, upon migratory responses of melanophores to environmental stimuli. It is possible, therefore, that the proximal area of the ventral fin does not provide an "attractive" stimulus for the migrating melanoblasts and that instead such cells migrate and complete their differentiation in the more "attractive" distal portion of the fin. Because of the peculiar melanophore distribution and because of the lack of other varieties of pigment cells in the tail, it is hard to explain the tail pigmentation patterns on the basis of physiological antagonisms between migrating chromatoblasts as has been suggested by Twitty and Niu (1954).

As an alternative explanation, it can be supposed that melanoblasts invade both the proximal and distal areas of the fin, but for reasons as yet unknown, only the distal portion of the tail provides a medium favorable for melanin synthesis. Wilde (1955) has strongly suggested that special metabolism of phenylalanine is required for normal differentiation of neural crest elements; thus, one might reason that prevalence of phenylalanine in the distal region may provide the stimulus for tail fin melanization. Furthermore, a gradient of this substance or some other melanogenic substance flowing outward from the somite to the edge

of the ventral fin would possibly account for the apparent differentiation gradient observed in the fin melanophores during normal development. Consistent with this hypothesis, the unpigmented margin along the edge of the regenerated fin may result from either a gradient which is too weak at its periphery, or from a diffusion blockage which does not allow penetration of the melanogenic substance to the edge of the fin.

Another fundamental question which suggests itself is concerned with the fact that not only are the tail melanophores extraordinarily light-sensitive but their responses are just the reverse of those of melanophores of other areas. The present observation that epidermal melanophores on the head and dorsal surface contract as a result of dark exposure is known for other amphibian larvae: for Ambystoma, Babak (1910) and Laurens (1917) and for Taricha and Rana, Bagnara (unpublished). To our knowledge, however, the very marked melanophore expansion, such as is seen in the tail of Xenopus, is unique among Amphibia. What mechanism allows the tail melanophores alone to possess this capacity is an enigma. Apparently the distal tail fin provides an environment which is favorable not only for melanogenesis but also for the development of a light-sensitive mechanism.

Whether the dorsal melanophores of Xenopus larvae are directly stimulated by light has not been determined; however, one is led to think that this is so because of the observations by Laurens (1917), who showed that the dorsal melanophores of blinded Ambystoma larvae behave toward light like those of intact animals. His additional observation that melanophore expansion after removal to light occurs much faster than the original melanophore contraction brought about by dark-treatment suggests that a photochemical reaction is involved in this system just as for the tail fin melanophores. Although light sensitivity of the dorsal melanophores is apparently a common thing among amphibian larvae, it should be emphasized that the reaction is relatively subtle, with changes of approximately 2 units of M. I. compared to a change of about 4 M. I. units for Xenopus tail melanophores.

Coincident with the unusual behavior of the tail melanophores of Xenopus is the unique form exhibited by these chromatophores. In an expanded state, their very thin branches lead one to suspect that they have less melanin than the thick melanophores on the dorsal surface. Perhaps this is a reflection of the apparent low sensitivity of the tail melanophores to the chromatotrophic hormone. Under normal lighting conditions, when the dorsal melanophores are expanded under influence of CTH, the contracted state of the tail melanophores implies that they are not sensitive to the circulating level of hormone. If the amount of hormone is raised by injection of CTH (Thing, 1952), the tail melanophores expand, strongly suggesting that the threshold level of response to CTH is higher for the tail melanophores than for those on the dorsal surface. That the tail darkening reaction is independent of hypophyseal stimulation was shown by Bagnara (1957) who pointed out that it occurs equally well in normal larvae, in hypophysioprivic larvae and in excised tails.

It is interesting to note that the response of the tail melanophores to light appears fairly early during the differentiation of these cells. Possibly it may even be present in melanoblasts before the synthesis of melanin. The reaction does not appear to be very strong in young melanophores; however, due to the relatively

small amount of melanin in these young cells, it could be fully developed and not appear so. It is significant that the melanophores of the regenerated tail can carry out the tail darkening reaction. This seems to indicate that the tail has regenerated completely, not only morphologically but physiologically as well.

Summary

1. The peculiar light-sensitive melanophores in the ventral fin of Xenopus larvae first appear about stage 47. Gradually, pigmentation increases in the distal half of the fin, but the proximal portion remains free of melanophores throughout the larval period. New melanophores first become visible at the fin edge, with an apparent gradient of melanophore differentiation from somite to edge. The young melanophores are thin and elongated, but as differentiation proceeds they add new projections and become stellate. Even the youngest melanophores exhibit some degree of tail darkening in the absence of light, but the strongest response is displayed by the fully formed melanophores.

2. Four days after tail extirpation, melanophores are seen in the regenerating ventral fin. Pigmentation returns to all of the ventral fin except for a margin along the fin edge, with a "somite-to-edge" gradient again apparent. The new melanophores are of the typical tail fin type and are fully light-sensitive.

3. Melanophores on the dorsal surface differ markedly from those on the tail. The former are heavily pigmented and are apparently more sensitive to the chromatotrophic hormone than the latter. Dorsal melanophores contract upon exposure to darkness and expand in the light. They are less reactive to changes in illumination than the tail fin melanophores.

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THE RELATIONSHIP OF REPRODUCTIVE TEMPERATURE AND THE GEOGRAPHICAL RANGE OF THE MARINE WOODBORER LIMNORIA TRIPUNCTATA[1]

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The objective of the study was to determine the influence of temperature on populations of the wood-destroying isopod *Limnoria*. The genus, which is economically important, contains about 16 described species (Menzies, 1957; Menzies and Becker, 1957; Pillay, 1957). Geographically, the species of the northern hemisphere have been divided into boreal, *L. lignorum;* temperate, *L. quadripunctata;* temperate-tropical, *L. tripunctata;* and tropical, 5–7 species.

The species we have worked with, *Limnoria tripunctata,* is found around the world. It is a hardy species which survives and reproduces under laboratory conditions. Our interest has been population growth at temperatures which might be encountered by the species in nature. On the basis of our results, we believe that we can explain the known distribution of the species in terms of the effect of temperature on population growth.

The specimens studied were collected through the courtesy of Mr. Thomas P. May, manager at the International Nickel Company testing laboratory at Wrightsville Beach, North Carolina, where the species is particularly abundant. The animals were air-shipped to the laboratory in containers which were packed so that oxygen was not excluded.

Procedure

The animals received at the laboratory were removed from the infected wood, and sorted for size and sex. For each culture, 25 males and 25 non-gravid females were placed in a dish containing sea water and a piece of presoaked pine. In all three experiments a total of 1050 animals was used. Non-gravid females were selected in order to keep each culture equivalent to the next. The use of females with eggs in different stages of development would have permitted errors in defining the reproductive temperature range and population growth at the various temperatures. In order to permit the animals to establish burrows in the wood, each culture was allowed to stand one week at room temperature (20–24° C.). Salinity was held within tolerable limits by addition of distilled water. Tolerable limits, 30–39‰ , were determined by preliminary experimentation. Salinity data for the three experiments are presented in Table I.

Seven cultures were prepared for each experiment in the manner described and one of each was put into a controlled temperature box and kept for a period of 66 days. This time duration was selected in order to allow for the production of young, but not their maturation. The cultures were kept at approximately

[1] Contrib. No. 395.

0, 5, 10, 15, 20, 25 and 30° C. Usual temperature variation in the cultures was ± 1° C. Short-term larger variations did occur as a result of equipment and power failure. These occasional failures resulted in a gradual return to room temperatures. For over 99% of the time, temperature variation was within ± 1° C. The temperature means from the twice daily record are shown in Table I.

TABLE I

Salinity of culture water at the start and finish of each experiment

Original salinity	Experiment I June, July, Aug. 35.7‰	Experiment II Nov., Dec., Jan. 34.1‰	Experiment III May, June, July 35.0‰
Temperature		End salinities	
0°	37.0‰	34.2‰	34.5‰
5	36.8	34.1	33.7
10	37.2	34.0	33.6
15	37.9	33.6	35.1
20	38.1	33.8	33.8
25	37.6	33.8	33.2
30	38.4	32.5	34.6

Mean temperatures during each 66-day period

Experiment I June, July, Aug.	0.5° C.	5° C.	9.9° C.	15.1° C.	20° C.	25° C.	29.8° C.
Experiment II Nov., Dec., Jan.	0° C.	5° C.	10° C.	15.1° C.	20° C.	25.1° C.	29.9° C.
Experiment III May, June, July	1° C.	6° C.	11° C.	16° C.	21° C.	24° C.	29.5° C.

The measurements taken of the young at the end of the 66-day period are recorded in size class, based on pleotelson width. The size classes used were taken from Johnson and Menzies (1956).

RESULTS

The results of three experiments, each of 66 days duration, are presented in tabular form (Tables II and III). These are the number of young present at the end of each experiment, the largest size attained by the young, adult mortality and gravidity of surviving females. From these data the population change in 66 days has been determined (Fig. 1).

Cultures kept at 0° C. showed no reproduction and the highest adult mortality (86–100%). At 0° C. the animals were immobile, did not feed and starvation is the probable cause of death.

The 5° C. cultures also showed no reproduction and a high adult mortality (50–92%). The animals were feebly motile and showed slight feeding, but here again starvation is the probable cause of death.

The 10° C. cultures showed no reproduction and a lower mortality (0–64%).

In two experiments gravid females were present; however, only eggs were found, and no larva or young were produced in the 66-day period. The animals were sluggish but did feed.

The 15° C. cultures showed a reproductive rate between 0 and 6 young produced per female. We are confident that a female produces more than one brood

TABLE II

Degrees Centigrade.....	0°	5°	10°	15°	20°	25°	30°
Adult mortality—Number of individuals in each culture of 50 animals that died in 66 days							
Experiment I June, July, Aug.	46	25	6	3	8	7	7
Experiment II Nov., Dec., Jan.	50	46	32	20	27	26	32
Experiment III May, June, July	43	28	0	4	2	0	32
Number of young present at the end of 66 days							
Experiment I June, July, Aug.	0	0	0	152	302	457	254
Experiment II Nov., Dec., Jan.	0	0	0	0	43	64	9
Experiment III May, June, July	0	0	0	20	45	73	0
Largest size class attained by young in 66 days*							
Experiment I June, July, Aug.	—	—	—	2	3	3	3
Experiment II Nov., Dec., Jan.	—	—	—	—	2	2	2
Experiment III May, June, July	—	—	—	2	3	3	—

* Pleotelson Width Size Classes after Johnson and Menzies (1956).

Class	Width in mm.
2	.28–.33
3	.35–.40

during her lifetime and therefore a 15° C. temperature permits population growth to occur. Adult mortality was 6–40%.

The 20° C. cultures showed a reproductive rate between 2 and 12 young per female. The average rate was 5 young per female and growing population is evident. Adult mortality varied between 4 and 54%.

TABLE III

Gravidity at the end of the experiments

Degrees C.	Experiment I June, July, Aug.			Experiment II Nov., Dec., Jan.			Experiment III May, June, July		
	No. females present	No. females gravid	Per cent gravid	No. females present	No. females gravid	Per cent gravid	No. females present	No. females gravid	Per cent gravid
0°	3	0	0	0	0	0	4	0	0
5°	9	3	33	1	0	0	12	0	0
10°	21	9	43	14	0	0	25	9	32
15°	16	9	56	16	0	0	22	1	5
20°	21	7	33	16	0	0	23	9	39
25°	22	7	32	18	1	6	25	9	32
30°	20	2	10	7	0	0	0	3	33

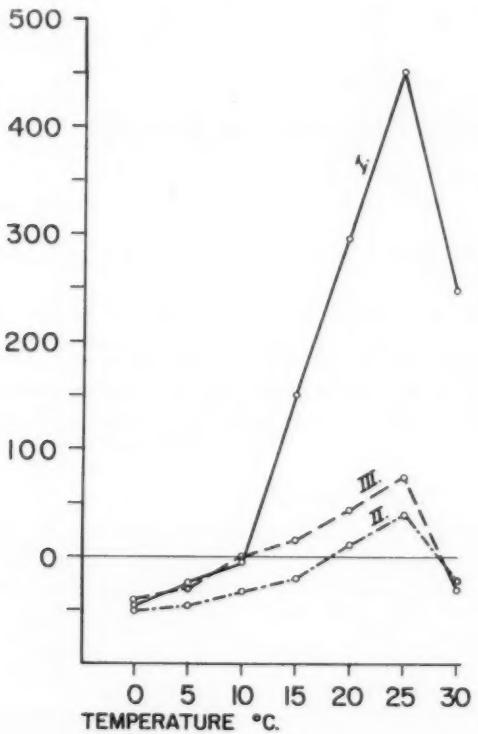

FIGURE 1. Population change at various temperatures after 66 days.

The 25° C. culture showed a reproductive rate between 3 and 18 young per female. The average rate was eight young per female, or about 1.6 times that of the 20° culture. The adult mortality rate was 0–52%.

The 30° C. culture showed a reproductive rate between 0.3 and 10 young per female. The average rate was three young per female. The adult mortality varied between 14 and 64%.

The most obvious feature of the three experiments is the lack of population growth below 10° C. The animals do not feed at these temperatures and eventually die of starvation as their fat reserve is depleted. The greatest population increase occurred in the cultures kept at 25° C. This might be considered the optimal temperature for population growth.

Adult mortality was highest at temperatures below 10° C. and at 30° C. Mawatari (1950) records 2–6° C. as the temperature lethal to adults of *Limnoria lignorum* (actually *L. tripunctata*). Our results also agree with the findings of Kampf (1957), who reports maximum population growth for *Limnoria tripunctata* from the Mediterranean to occur at 24° C. He also reports a shortening of the life span at higher temperatures which may account for the higher mortalities at 30° C.

Inherent Seasonal Rhythm

Kampf (1957) reported a cyclic occurrence of gravidity for *Limnoria* kept under reasonably constant aquarium conditions. Unfortunately, his populations were mixed cultures of two species, *L. carinata* and *L. tripunctata*. Nevertheless, the low proportion of the former in his culture probably means that the cyclical record of gravidity is real for *L. tripunctata*. Coker (1923) reports that there is little winter gravidity found in Beaufort, North Carolina, *Limnoria* during the winter. Our observations of samples shipped from Wrightsville Beach, North Carolina, confirm this. The fact that culturing the specimens in the laboratory at temperatures approached only during the summer in the field still results in low gravidity and low young production suggests that we are dealing with an endogenous seasonal gravidity period which does not appear to be subject to immediate modification by change of temperature, and a reproductive rate imposed on the former which is subject to modification by change of temperature. It will be of the greatest interest to determine whether a seasonal endogenous reproductive period occurs in populations of the same species collected from a tropical locality where seasonal temperature fluctuations are almost absent.

Distribution, Temperatures and Population Growth

From the data presented, it would be a likely prediction that *Limnoria tripunctata* should not occur in localities where the sea water temperature is below 10° C. for most of the year. On the Atlantic coast the species is known to occur as far north as Cape Cod, Massachusetts. Here the yearly average sea water temperature is about 11° C. For five months of the year, however, the temperature rises above 15° C. and thus there is time for the populations to reproduce. The existence of the species is probably very precarious at this locality since monthly averages are below ten degrees for five months of the year. While signs

of *Limnoria* damage are extensive in the area, more often than not the burrows are empty and finding infested wood is sometimes a difficult job. North of Cape Cod, at Eastport, Maine, for example, the sea water temperature reaches 10° C. for only three months of the year. In such a situation we would not expect *Limnoria tripunctata* and to our knowledge the only species found there is *Limnoria lignorum*.

On the Pacific Coast of North America the species is not known as far north as Friday Harbor, Washington. Here the mean temperature of the surface sea water rises to 10–11° C. for only three months out of the year. During the re-

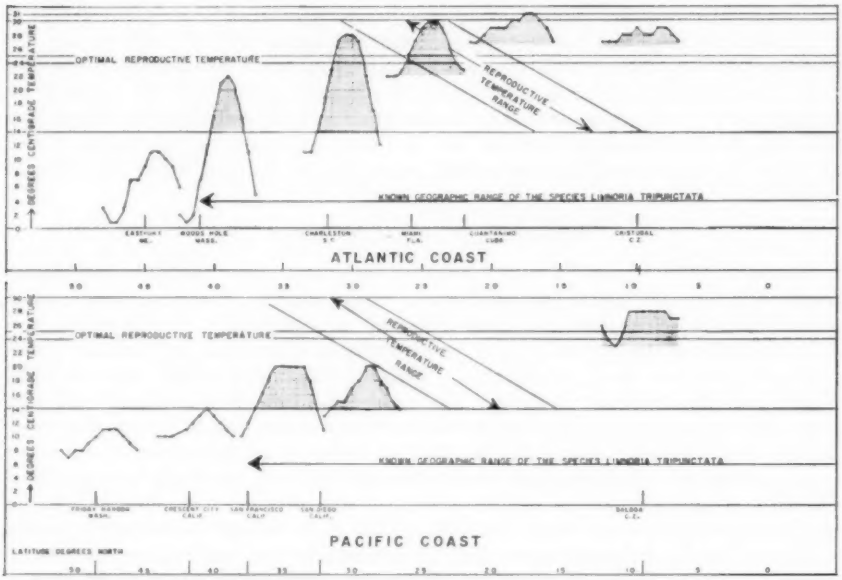

FIGURE 2. Geographical distribution, mean monthly surface sea water temperatures plotted at approximate longitude and reproductive temperature limits (the horizontal lines connected by the sloping block): The shaded areas represent the period of time that the temperature is within the reproductive range of the species. The minimum reproductive temperature, 14° C., is estimated from the average of the three experiments to be at this temperature. At this point one young would be produced for each adult.

maining months it is lower than 10° C. This would give the species three months to feed, and no period of time suitable for reproduction. Southward, the species is also absent at Crescent City, California. Here temperatures rise above 10° C. for ten months of the year. The maximum temperature is 13° C. and this occurs for only two months of the year; 15° C. is not reached seasonally.

The species occurs along the Pacific Coast from San Francisco, California, to Mazatlan, Mexico, and probably as far south as Panama. At San Quentin, San Francisco Bay, lethal temperatures do not occur seasonally and temperatures favor-

ing population growth occur for seven consecutive months of the year. Seasonal temperature distribution, reproductive temperature limits for the species, and the established geographic distribution of *Limnoria tripunctata* in North America are shown in Figure 2. The remarkable coincidence between the geographic range of the species and the seasonal occurrence of temperatures favorable for reproduction leads us to conclude that temperature is a major factor controlling the distribution of the species.

The reason for the restriction of a species to a given geographic range has been of interest to zoologists and zoogeographers for many years. Temperature means, maxima and minima have been examined as parameters of possible control. The duration of a given range of temperatures has also been considered (Menzies and Hedgepeth, unpublished data). Experimentally determined physiological lethal limits for short periods of time are usually too wide to account precisely for the distribution of a marine species. Our examination of the effect of temperature on population growth of a single marine species suggests that the geographic distribution of this species is closely tied to the prevalence of temperatures favoring reproduction. The minimum duration of a favorable temperature necessary would depend on the growth rate of the species. Doubtless the duration of unfavorable temperatures for survival is also important. In terms of our experimental data it is difficult to explain the survival of populations of *Limnoria tripunctata* at Woods Hole, where it is necessary for the species to maintain itself for three months of the year at mean temperatures below 5° C. Our data suggest that this is a remote possibility; nevertheless, the species is precariously established.

The figures were drawn by Carolyn Peppin and Don Robinson. This research was sponsored by the U. S. Navy, Office of Naval Research, Contract Nonr-266 (41).

Summary

1. Experimental results show that *Limnoria tripunctata* will feed at temperatures ranging from approximately 10° C. to 30° C. The reproductive temperature range appears to be from about 15° C. to 30° C. and the greatest population increase is in the neighborhood of 25° C. Excessive mortality results at 30° C.

2. Gravidity and hence number of young produced depends upon season and does not appear to be immediately modified by the presence of favorable reproductive temperatures.

3. The experimental results agree well with the known geographic range of the species.

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THE LARVAL STAGES OF PLEURONCODES PLANIPES STIMPSON (CRUSTACEA, DECAPODA, GALATHEIDAE)

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The zoeal larval stages of a galatheid crab were found in large numbers (up to 42,000/1000 m.^{3}) in plankton tows taken at several stations of the Marine Life Research program of the California Cooperative Oceanic Fisheries Investigations near Magdalena Bay, Baja California, Mexico. Since these unknown larvae were found in an area where the galatheid *Pleuroncodes planipes* was extremely abundant, it seemed likely that they were the zoeal stages of that species.

Methods

The larvae were tentatively identified as *P. planipes.* This identification was later confirmed by two methods: (1) the first larval stages were hatched from the eggs of the adult *P. planipes,* (2) one specimen of the fifth larval stage was caught in the process of molting to the juvenile stage, and this juvenile could be identified as *P. planipes.* Several attempts were made to follow the succession of larval molts in the laboratory. Hatching the larvae from the egg was relatively easy, and was achieved by several methods. However, no larvae survived longer than eight days, and all died before they had molted once. Plunger jars and still-water aquaria were used with variations of temperature, aeration and food (*Platymonas, Chlamydomonas* and Coccolithophores). The plunger jar method and a procedure described briefly by Ray (1958; p. 501), involving rotating flasks, apparently failed because of the constant vigorous tumbling of the larvae.

Adult females carrying eggs have been found in the plankton from December through March. Gravid females kept in the laboratory hatched larvae during most of that period and the same is probably true of females in nature. As larvae of all stages are found in the plankton from January to July, it has proved impossible to determine rates of development.

Description of the Larvae

Stage I (Figure 35)

First antenna (Fig. 1). This appendage has a long, unsegmented base with two distal branches. These, the exopodite and endopodite, are continuous with the base. The former bears six setae, the latter, a long plumose spine.

Second antenna (Fig. 6). The antenna consists of a base, continuing into a long medial prickly spine, and a scale. The scale bears about eight plumose setae and is continued as an apical spine, prickly at the terminal end. On the

[1] Contribution from the Scripps Institution of Oceanography, New Series.

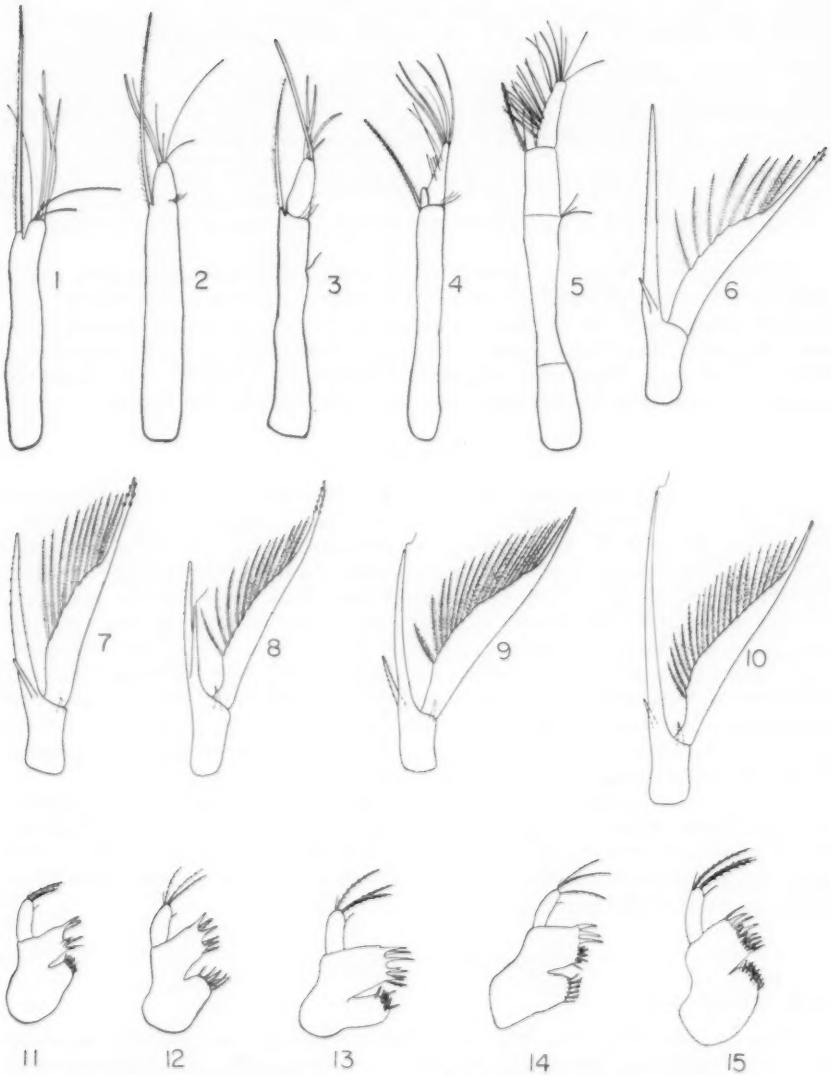


FIGURE 1. First antenna, Stage I.
FIGURE 2. First antenna, Stage II.
FIGURE 3. First antenna, Stage III.
FIGURE 4. First antenna, Stage IV.
FIGURE 5. First antenna, Stage V.
FIGURE 6. Second antenna, Stage I.
FIGURE 7. Second antenna, Stage II.
FIGURE 8. Second antenna, Stage III.
FIGURE 9. Second antenna, Stage IV.
FIGURE 10. Second antenna, Stage V.
FIGURE 11. First maxilla, Stage I.
FIGURE 12. First maxilla, Stage II.
FIGURE 13. First maxilla, Stage III.
FIGURE 14. First maxilla, Stage IV.
FIGURE 15. First maxilla, Stage V.

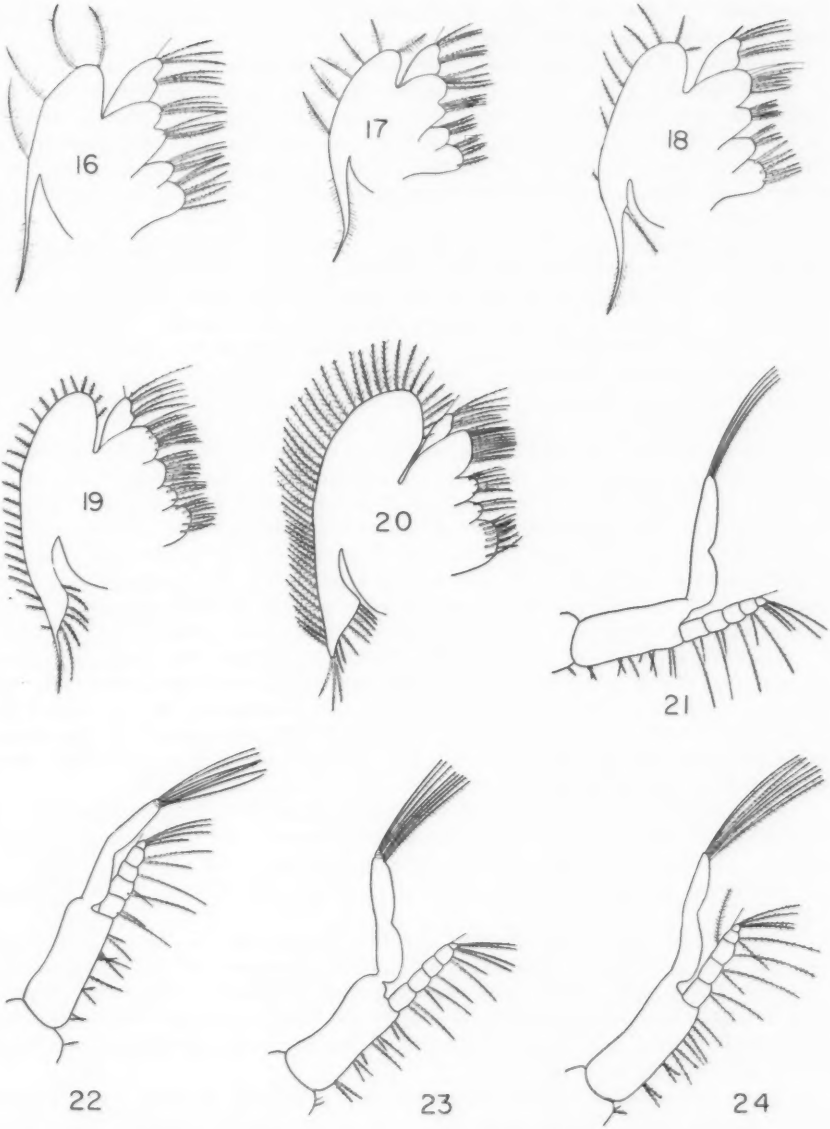


FIGURE 16. Second maxilla, Stage I.
FIGURE 17. Second maxilla, Stage II.
FIGURE 18. Second maxilla, Stage III.
FIGURE 19. Second maxilla, Stage IV.
FIGURE 20. Second maxilla, Stage V.
FIGURE 21. First maxilliped, Stage I.
FIGURE 22. First maxilliped, Stage II.
FIGURE 23. First maxilliped, Stage III.
FIGURE 24. First maxilliped, Stage IV and V.

dorsal side of the inner flagellum is a smooth spine which, though small in this stage, increases in size in later stages.

Mandible. The mandible is a simple toothed process lacking a palp.

First maxilla (Fig. 11). The endopodite is a simple unjointed structure bearing three spinose setae and two smooth setae. The endopodite does not change in number of setae but only in size in the next four stages. The exopodite is split into a basipodite and a coxopodite. These generally add setae in the later stages. In Stage I, the basipodite bears five heavy setae. The coxopodite has seven setae.

Second maxilla (Fig. 16). As in the endopodite of the first maxilla, the endopodite of the second maxilla does not change the number of setae in any of the stages. Terminally on the endopodite there are four setae; three spinose and one naked. A group of three spinose setae is located subterminally. The basipodite lobes each bear four spinose setae, as does the distal lobe of the coxopodite. The proximal lobe of the latter carries six spinose setae. The scaphognathite carries only four plumose setae along its margin. Its posterior end is produced into a long plumose projection.

First maxilliped (Fig. 21). The maxilliped is well developed in the first larval stage. The appendage consists of a coxopodite with one seta on its interior margin, an unsegmented basipodite, an endopodite with five segments, and an exopodite with two indistinct segments. The basipodite has ten spinose setae on its interior margin. There are four setae terminally on the exopodite. The five segments of the endopodite each bear spinose setae, but in differing numbers. The fourth, second, and first segments have two setae, and the third but one seta. There is also one fine, smooth seta coming off from the joint of the distal (fifth) segment.

Second maxilliped (Fig. 25). The second maxilliped has much the same structure as the first. The endopodite of the second maxilliped, however, has only four segments; the third is elongated. The coxopodite has no setae; the basipodite has three spinose setae. The terminal (fourth) segment of the endopodite has four spinose setae and one fine, smooth seta. There are four setae terminally on the exopodite.

Third maxilliped (Fig. 29). The third maxilliped is rudimentary in Stage I; it lacks setae and is poorly developed in musculature. The appendage consists only of a basipodite and an exopodite; the endopodite is not present.

Abdominal segments. There are five segments, plus the telson in Stages I and II.

Telson (Fig. 36). The telson of the first larval stage has seven projections on each side. The first (outermost) is a spinose extension of the telson, and is unjointed at its base. This spine has 6–10 thorns distally. The second projection is a fine, hair-like, plumose seta. The next five are similar in that they are all plumose setae. In addition to the plumes, each seta carries thorns on its inner and outer margins.

Along the posterior margin of the telson between the plumose setae, several small thorns occur. In Stage I larvae there are usually one to three thorns between each of the inner five setae. Generally the number of thorns increases between the setae more medially located, so that there are usually two to three thorns between setae #6 and #7. In the medial cleft of the telson, one to three thorns

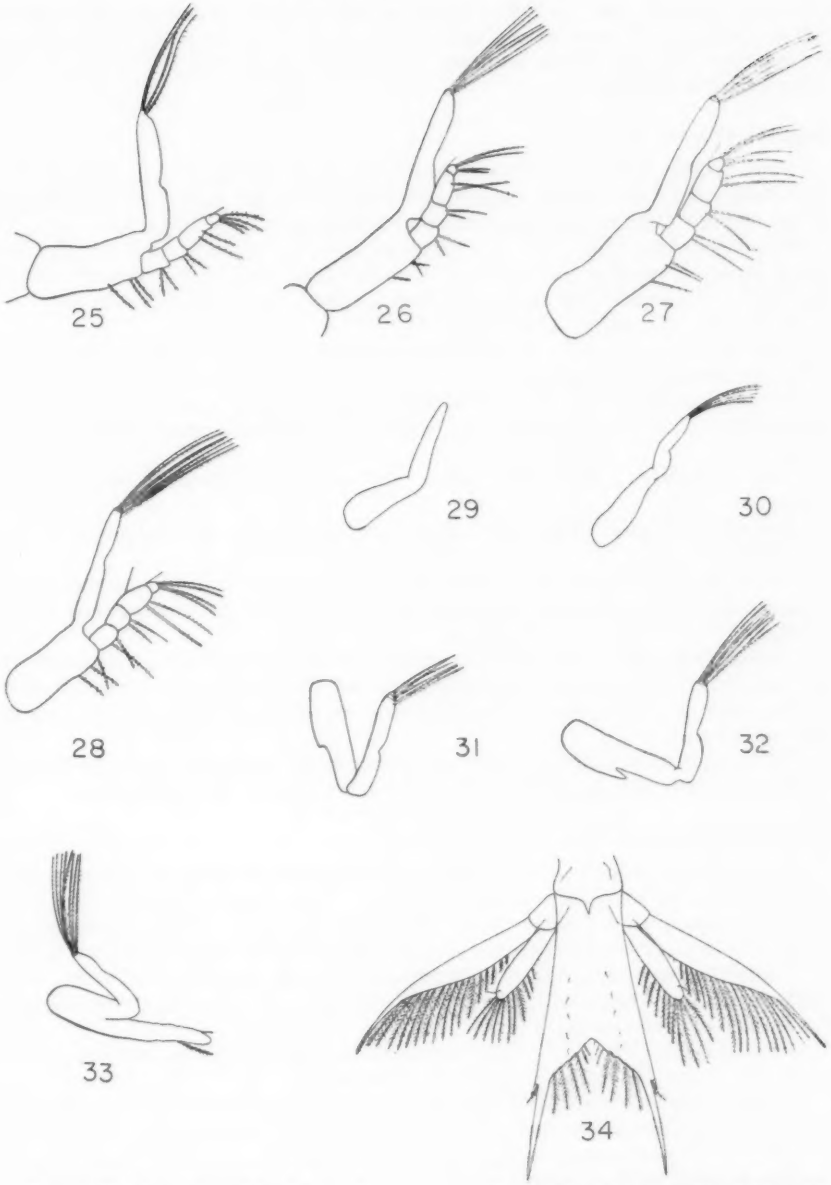


FIGURE 25. Second maxilliped, Stage I.
FIGURE 26. Second maxilliped, Stage II.
FIGURE 27. Second maxilliped, Stage III and IV.
FIGURE 28. Second maxilliped, Stage V.
FIGURE 29. Third maxilliped, Stage I.
FIGURE 30. Third maxilliped, Stage II.
FIGURE 31. Third maxilliped, Stage III.
FIGURE 32. Third maxilliped, Stage IV.
FIGURE 33. Third maxilliped, Stage V.
FIGURE 34. Telson, Stage IV.

may occur on each side. A tuft of short fine hairs occurs further medially. Each tuft is composed of seven to ten hairs. The numbers of these thorns and hairs are variable and are seldom identical between the right and left halves of the telson of any individual.

Stage II (Figure 37)

First antenna (Fig. 2). This appendage is much the same as in Stage I, but here three small hairs appear toward the distal end of the base. These hairs are on the location of an ultimate segmentation appearing fully in Stage III.

Second antenna (Fig. 7). The antennal scale has gained two more plumose setae, giving it usually ten. A new spine has appeared on the ventral side of the base. This spine is not segmented from the base, but is common with it.

Mandible. No change from Stage II.

First maxilla (Fig. 12). A seta has been added to the basipodite. The coxopodite has remained unchanged.

Second maxilla (Fig. 17). The endopodite, basipodite and coxopodite are unchanged. The scaphognathite has added two marginal setae, giving a total of six.

First maxilliped (Fig. 22). There are now seven terminal setae on the exopodite.

Second maxilliped (Fig. 26). There are now seven terminal setae on the exopodite.

Third maxilliped (Fig. 30). This appears to have become a functional organ; its musculature has developed and there are six setae on the terminal end of the exopodite.

Telson (Fig. 38). The primary change in the telson of Stage II larvae is the addition of one plumose seta, increasing the number of projections to eight. In this stage, however, the third projection does not have thorns on its margins, but only plumes.

The arrangement of thorns occurring between setae is similar to that of Stage I. In Stage II, however, there are neither hairs nor thorns in the medial cleft.

Stage III (Figure 39)

First antenna (Fig. 3). The segmentation initiated in Stage II has been completed, setting the exopodite off from the base. Three hairs originate at the joint. A hair appears on the exterior lateral margin of the base. The combined length of the new terminal segment and the setae is longer than the spine of the endopodite.

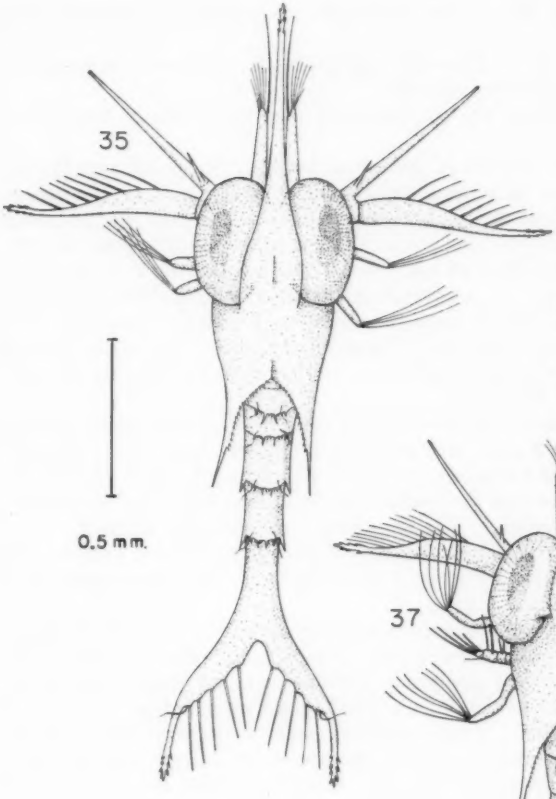
Second antenna (Fig. 8). The antennal scale now usually has 11 setae. The spine on the inner flagellum has increased in length and now has a terminal hair.

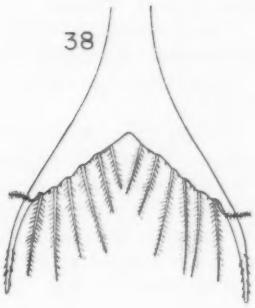
Mandible. No change.

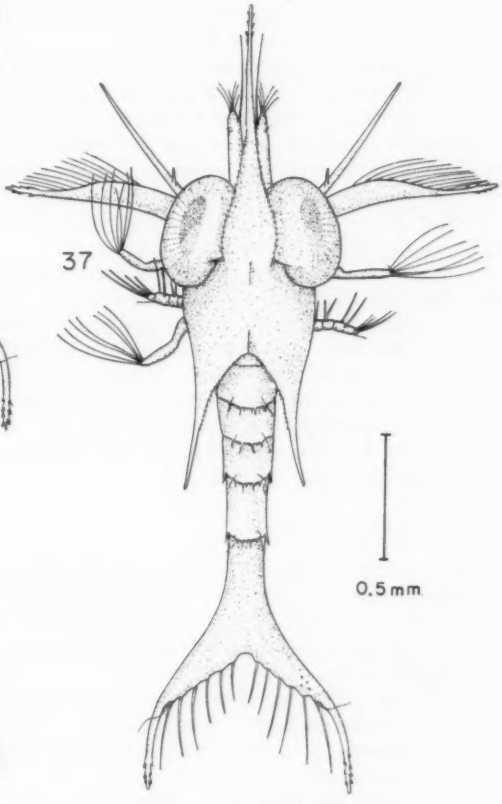
First maxilla (Fig. 13). The basipodite has eight setae, an increase of two setae over Stage II. The coxopodite is unchanged.

Second maxilla (Fig. 18). The distal lobe of the basipodite now has seven setae. The proximal lobe has six. The coxopodite is unchanged. The exterior margin of the scaphognathite now has nine setae, plus one seta located on the interior margin of the posterior end.

First maxilliped (Fig. 23). There are now eight setae on the exopodite.







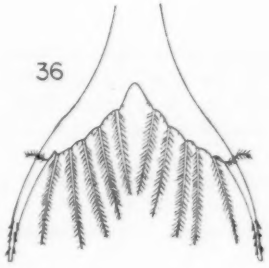


FIGURE 35. Larva, Stage I.
FIGURE 36. Telson, Stage I.

FIGURE 37. Larva, Stage II.
FIGURE 38. Telson, Stage II.

Second maxilliped (Fig. 27). The exopodite has added one terminal seta, giving a total of eight.

Third maxilliped (Fig. 31). The only change in the third maxilliped is the development of a small bud on the basipodite.

Abdominal segments. There are six segments, plus the telson in Stages III, IV and V.

Telson (Fig. 40). The changes in the telson between Stages II and III are radical. There are typically now nine projections on each half of the telson. The first (outermost) is a short naked spinose extension of the body of the telson. The second is a plumose hair-like seta. The third is a short heavy plumose seta. The fourth is the major projection of the telson; it is an extension of the telson and is not jointed at its base. It is covered on its interior and exterior margins by many small thorns. The fifth through the eighth projections are plumose setae bearing thorns on their lateral margins. The ninth projection is a small plumose seta lacking thorns. This innermost seta is not always present. It may be lacking on one or both sides of the telson.

Thorns are found abundantly between the fourth through the eighth projections. Between projections #4 and #5, zero to three thorns may occur. Usually three to five thorns are found between each of the fifth through eighth projections. Between the eighth projection and the medial line up to four hairs may be present, in addition to the ninth projection. The occurrence of these hairs is erratic.

On the dorsal surface of the telson, two pairs of very fine setae are found. The posterior pair is near the base of the seventh projection. The second pair is in line with the first pair, but anterior to it.

Uropods. The inner uropods are present only as poorly developed plates; they carry no setae. The outer uropods have from 8 to 11 plumose setae. The setae do not carry thorns. Their number is variable and may differ between the right and left sides. The tip of the outer uropod is not produced in an extended spine, but is short and naked. Small accessory setae commonly occur on the ventral surface of the outer uropod. These are not at the margin but are based somewhat anteriorly of the posterior margin. Generally there are two on each uropod, one between setae #1 and #2 (counting from the distal end) and the second between setae #4 and #5.

Stage IV (Figure 41)

First antenna (Fig. 4). The exopodite has gained three pairs of setae along its inner margin. In addition, the endopodite has developed a joint at its base.

Second antenna (Fig. 9). The scale usually now has 17 plumose setae. The spine on the inner flagellum has now increased in length so that it is much longer than the flagellum.

Mandible. The mandible at this stage has a small bud on the anterior margin which will become a palp in Stage V.

First maxilla (Fig. 14). The basipodite has 11 setae. The coxopodite has 10–11 spinose setae.

Second maxilla (Fig. 19). The lobes of the basipodite and coxopodite have added setae, but their absolute numbers are variable. The exterior margin of the scaphognathite now has about 25 setae; the posterior interior margin has six setae.

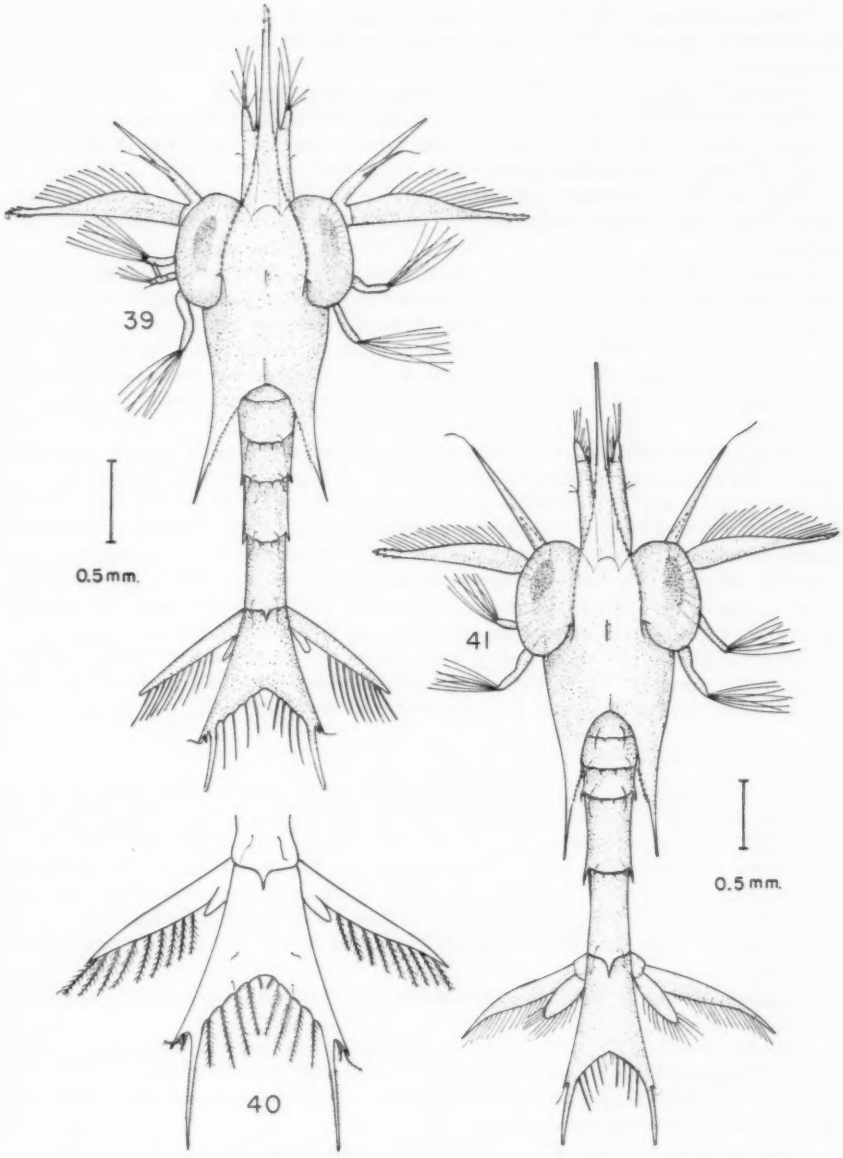


FIGURE 39. Larva, Stage III.
FIGURE 40. Telson, Stage III.
FIGURE 41. Larva, Stage IV.

First maxilliped (Fig. 24). The only change from Stage II and III is in the addition of a plumose seta on the exterior margin of the third segment of the endopodite.

Second maxilliped (Fig. 27). No change from Stage III.

Third maxilliped (Fig. 32). The exopodite has added two setae, giving now a total of eight. The bud on the basipodite has somewhat increased in size from Stage III, but is still rudimentary.

Thoracic legs. Thoracic legs are not visible on Stage IV larvae from a lateral view. They are present, however, as poorly developed appendages under the carapace.

Telson (Fig. 34). The telson in this stage is similar in aspect to that in Stage III, but it has added several plumose setae. The total number of projections may vary from 10 to 12, with the deletions occurring in the innermost setae; *i.e.,* setae #11 and #12 may individually or both be absent. Typically there are 11 projections. Setae #9, #10, #11, and #12 are plumose but do not bear thorns. Projection #1 appears to be jointed and is no longer common with the body of the telson.

On the dorsal surface of the telson there are now five pairs of fine, hair-like setae. Three pairs have been added anteriorly to those present in Stage III.

Uropods. Inner uropod: this now has from 10 to 17 plumose setae. Three tufts of hairs occur medially from the innermost seta. A fine, hair-like accessory seta is found on the dorsal surface of the distal end of the inner uropod. Outer uropod: the extremity of this branch is now extended as a long spine bearing many thorns along its lateral margins. From 13 to 21 plumose setae are present on the posterior margin of this branch. Several fine hairs are found medially of the innermost seta. Four accessory setae are again found on the ventral surface of the outer uropod. These commonly occur between setae #1 and #2, #4 and #5, #9 and #10, and #14 and #15, starting from the outer seta.

Stage V (Figures 42–44)

First antenna (Fig. 5). The exopodite has added more medial setae, bringing the total to about 22. The endopodite has elongated, but is still shorter than the exopodite. The base now appears to be very lightly segmented into three parts.

Second antenna (Fig. 10). The number of plumose setae on the scale has increased to usually 19. The inner flagellum is reduced to a very small size and the spine which formerly grew upon it is as long as the antennal scale.

Mandible. The palp on the anterior margin is well developed, though unsegmented.

First maxilla (Fig. 15). The basipodite has about 15 setae and the coxopodite has about 13 setae.

Second maxilla (Fig. 20). The basipodite and coxopodite have added setae. The scaphognathite now has about 43 plumose setae on its exterior margin. Its posterior interior margin now has eight setae. The extreme posterior spine has been replaced by three plumose setae.

First maxilliped (Fig. 24). Unchanged from Stage IV.

Second maxilliped (Fig. 28). The only change from Stage III and IV is in the addition of a smooth seta on the exterior margin of the third segment of the endopodite.

Third maxilliped (Fig. 33). At this stage the endopodite appears. However, it is soft, poorly musculatured and unsegmented. On its interior margin is a plumose seta and on its exterior margin, distally, is a fine smooth seta.

Thoracic legs. The thoracic legs are easily visible in a lateral view of the Stage V larvae. The first is chelate, the fifth is quite small and hidden behind the fourth leg.

Pleopods. There are now four pairs of unequally bifid pleopods present.

Telson (Fig. 43). The telson of this stage has reached its full development and variability of spine count is reduced. There are now commonly 12 projections. Of the inner eight projections, only #5 through #8 have thorns as well as plumes on their margins. One to five thorns are found on the margins of the telson between each of projections #4 through #9. No thorns are found medially of the ninth projection, but up to three hairs may occur between each of the setae between the ninth projection and the medial line. The five pairs of fine dorsal accessory setae of Stage IV are unchanged in Stage V.

Uropods. Inner branch: the inner branch now has from 18 to 21 plumose setae. Several hairs are found medially of the innermost setae. The fine setae occurring on the dorsal surface of the distal tip of the inner uropod in Stage IV is still found in this stage. Outer branch: the characteristic form of the outer uropod has not changed from that of Stage IV. The tip of the uropod is still drawn out as a spine and still bears thorns on its lateral margins. In this stage there are from 20 to 23 plumose setae on the posterior margins of the outer uropods. Fine, accessory setae occur ventrally between #1 and #2, #4 and #5, #9 and #10, and #14 and #15.

The Larval Stages

The five larval stages may be separated by the following key:

A. Uropods absent

1. The posterior margin of the telson has five plumose setae on each side; the third maxilliped is rudimentary. *STAGE I*
2. The telson has six plumose setae on each side; the third maxilliped is functional. *STAGE II*

B. Uropods present

1. The inner uropods are naked; pleopods are absent. *STAGE III*
2. The inner uropods have setae; pleopods are absent. *STAGE IV*
3. Pleopods are present. *STAGE V*

The sizes (total lengths) of the larvae in each stage were as follows (range and mean):

Stage	Minimum	Mean	Maximum	Number measured
I	2.6 mm.	2.7 mm.	2.8 mm.	8
II	3.1	3.4	3.6	8
III	4.1	4.6	5.0	7
IV	5.3	5.7	6.5	11
V	7.2	7.6	7.8	11

All the larvae measured and figured were from the Magdalena Bay area.

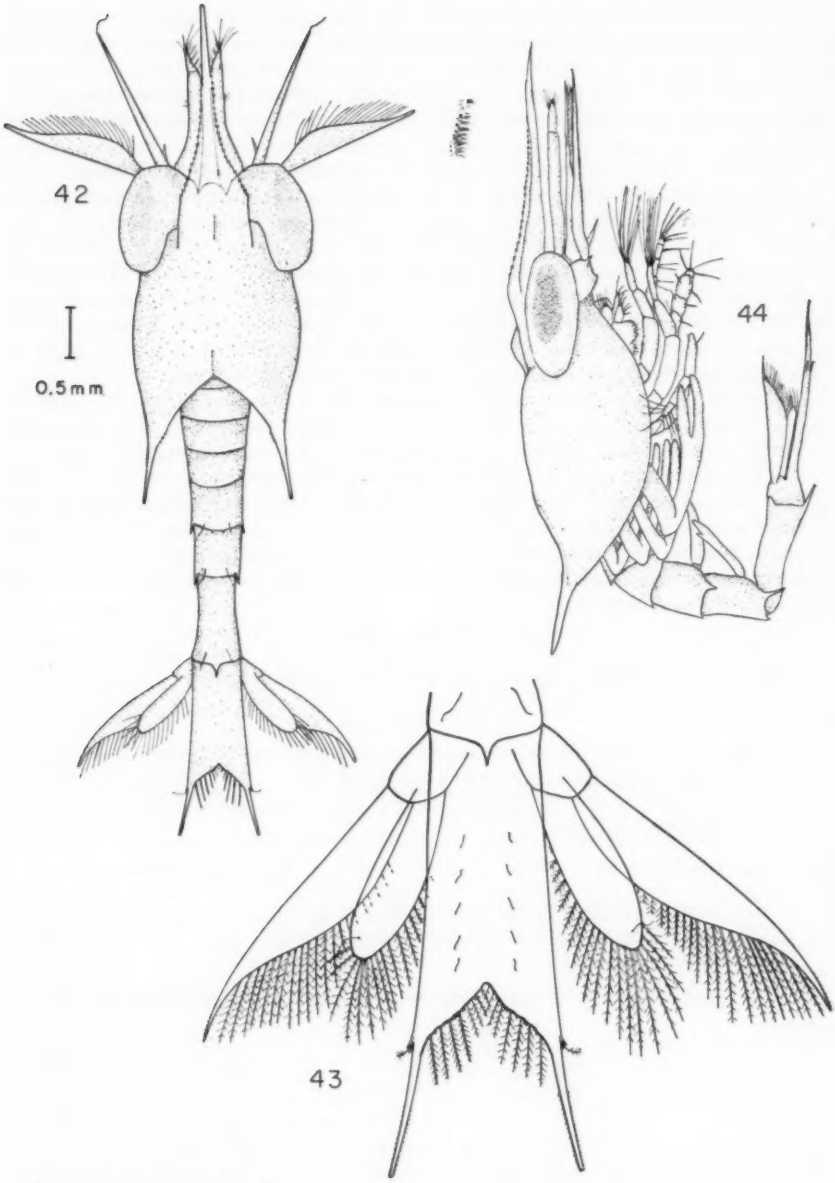


FIGURE 42. Larva, Stage V.
FIGURE 43. Telson, Stage V.
FIGURE 44. Larva, Stage V.

Lebour (1930) notes that *Galathea dispersa* may omit either Stage IV or V, but that there are usually five stages. It is not known if all *P. planipes* larvae pass through each of the five stages before molting to the juvenile stage; one or more stages may be by-passed. However, the measurements given above indicate that this is infrequent.

Discussion

All except one of the developmental features of *Munida* and *Galathea* larvae as described by Lebour (1930; p. 177) are also found in *P. planipes:* the pleopods do not appear as small knobs in the third stage in *P. planipes,* but appear without previous indication in the fifth stage. *Pleuroncodes* larvae share the following traits with larvae of *Munida,* but not with those of *Galathea:* the endopod of the first maxilla has only one segment; in Stages I and II the lateral-most spine of the telson of both *Munida* and *Pleuroncodes* is longer than any of the posterior margin on the telson; a large dorsal spine occurs on the posterior margin of the sixth abdominal segment in Stages III, IV and V. *Pleuroncodes* thus appears to be more closely related to *Munida* than to *Galathea.*

The larval stages of five species of the genus *Munida* have been described. G. O. Sars described the larvae of *M. sarsi* Brinkman in 1889. Johan Huus (1934) found that these were morphologically identical with the larvae of *M. tenuimana* M. Sars, with the exception of some minute hairs in the medial cleft of the telson. Lebour (1930) described the larval stages of *M. rugosa* (Fabricius) and later (1931) set up a key to distinguish the galatheid larvae of the Plymouth area. *M. rugosa* is, however, the only species of the genus *Munida* covered by this key. (Lebour described the larvae as *M. banffica;* Zariquiey (1952), however, has synonymized this species with *M. rugosa.*) Rayner (1935) described five larval stages of *M. gregaria* (Fabricius) and *M. subrugosa* (White) but was unable to distinguish the larvae of the two species. The adults of these two species are limited to the southern hemisphere and along the Eastern Pacific they do not occur north of 41° 30′ S. (Matthews, 1932; p. 470).

Larvae of *Pleuroncodes planipes* can be easily distinguished from the described larvae of European munids by the aciculate second antennae and the elongated postero-lateral spines of the carapace of the latter. They are, however, very much like the larvae of the New World munids described by Rayner (1935). The chief difference is the lack of serration on the lateral margins of the rostrum of the larvae of *M. gregaria* and *M. subrugosa.* The rostrum of each of the stages of *P. planipes,* except the first, is strongly serrated. The fifth stage of the *Munida* species has some serration on its rostrum, but not to the degree found in *P. planipes.* Rostral serration probably will not hold good in the future as a valid characteristic setting off the genus *Pleuroncodes,* for Gurney (1942; p. 255) mentions an unidentified galatheid larva from Bermuda which had a serrated rostrum. No species of *Pleuroncodes* is known from that area.

Both Huus (1934) and Lebour (1930, 1931) use color and arrangement of pigmented areas as an aid to specific identification. All color fades, however, from specimens kept longer than a few weeks in either alcohol or formalin. No color pattern was noted in *P. planipes* for that reason.

The area off Magdalena Bay (24° 40′ N., 112° W.) appears to be peculiar as far as *P. planipes* larvae are concerned. Within a 50-mile radius of this bay,

densities up to 42,000/1000 m.^{3} have been observed and are not uncommon. Outside this area, but well within the usual distributional area of adult *P. planipes*, the larvae, as sampled with a meter net from 140 m. to the surface, have an average density of 5/1000 m.^{3} (based on 22 tows taken between January and July; range 0–26/1000 m.^{3}). On this evidence it would appear that the Magdalena Bay area is a center of breeding. This is somewhat unusual in that adults are considered epi-pelagic and are distributed over a very wide range, from about 16° N. to 36° N. and from the littoral to several hundred miles off-shore.

The author wants to express his thanks to Dr. Martin W. Johnson for the help he has given me in this study. The author is also grateful for the assistance of Dr. E. W. Fager and Mrs. Margaret K. Riedel in criticism and preparation of this paper.

Summary

The larval stages of a galatheid decapod crustacean, *Pleuroncodes planipes*, have been identified, described and figured. There are five stages. First stage larvae were hatched from eggs carried by adults; no larvae molted under laboratory conditions. Larval morphology indicates that the genus *Pleuroncodes* is more closely allied to the genus *Munida* than to *Galathea*. The larvae occur in high densities (42,000/1000 m.^{3}) in the region of Magdalena Bay, Baja California, Mexico.

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THE MOULTING CYCLE IN BALANUS BALANOIDES L.

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Darwin, as early as 1854, noticed that a number of operculate cirripedes often moulted their skin, which included the exuviae of prosoma, appendages, penis, oesophagus, rectum and the inner lining of the mantle. He also stated that the frequency of moulting was related to the speed at which the barnacles grew.

Recently Costlow and Bookhout (1953, 1956) studied the frequency of moulting in relation to growth in recently settled cyprids of *Balanus improvisus* and *Balanus amphitrite*, but, since their observations were limited to a certain length of time after settlement, no accurate and detailed information is available regarding the seasonal fluctuations in the moulting rhythm and the factors controlling it.

The following investigation was therefore carried out to study the moulting and the factors influencing it in the boreo-arctic species *Balanus balanoides* L.

Method

Suitable groups of *Balanus balanoides* L. were obtained for the investigation on the shells of living *Mytilus edulis* growing on the piles of Bangor Pier in the Menai Straits, North Wales. In such a position, with a good current of water flowing past, both mussels and barnacles obtain abundant food and grow well. As soon as possible after collection all barnacles other than *B. balanoides* were removed. Usually 10–20 mature individuals were left on each shell. Care was taken not to perforate the shells of any specimens which were to be used in the experiment, since even a slight puncture, which can readily be made at the basal attachment, may lead to the death of the individual in a few days. During the first few days individuals which died were removed from the group and were not considered in the experiment.

Each group, comprising 20–30 individuals, was kept in 6-inch diameter glass crystallising dishes and the water was changed every day. It was not convenient to simulate the semi-diurnal tide; however, it was found beneficial for the barnacles to be out of water for about 8 hours each day. The dishes therefore were filled at night and emptied each morning and the cast skins present were counted. Sometimes during the breeding season pools of sperms were seen, probably indicating that copulation had occurred. Occasionally, during the period of liberation, swarms of nauplii were noted. However, if liberation occurred soon after dark, the larvae did not swarm to the side of the dish but were often devoured by the parents by the following morning. Large numbers of chocolate-red faecal pellets, containing much larval pigment and undigested chitinous appendages, were produced in consequence of this cannibalistic meal. These served to indicate that liberation had taken place. The presence of sperm pools and the evidence of liberation of nauplii were carefully noted whenever they occurred.

In some of the experiments the barnacles were fed liberally on *Artemia* larvae, and in others no food was given, a change of water being made only once a day. Both the fed and the starved animals were maintained at a series of temperatures. One series was kept in a thermostatically controlled refrigerator at $3 \pm 1°$ C., a second in a thermostatically controlled cool cabinet at 5 to 7° C., a third set in a cool basement, the temperature of which differed by only 1–2° C. from that of the mean sea water temperature, and a fourth in a laboratory where the temperature remained considerably higher, ranging between 15–21° C.

In order to study the influence of the parasite *Hemioniscus balani* on the moulting rhythm, infected specimens of *B. balanoides* L. and of *Elminius modestus* Darwin were collected from Brixham, S. Devon, where a high level of infection occurs (Southward and Crisp, 1954). Infected specimens of *B. amphitrite* var. *denticulata* Broch and *B. perforatus* Bruguière were collected from the warm water docks at Swansea and from the coast of the Gower Peninsula, respectively. For these experiments animals were isolated in dishes and fed on *Artemia* larvae; subsequent examination revealed whether they were infected.

Seasonal Variation in the Moulting Rhythm

Darwin (1854) in his monograph on Cirripedia mentioned that he was informed by a Mr. Peach that off the coast of Cornwall barnacle exuviae were most abundant in April–May and again in September. The specimens sent to Darwin included

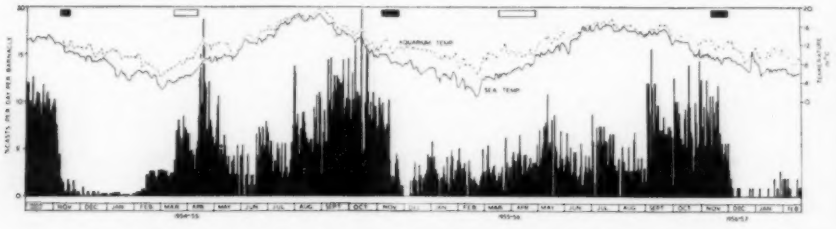

Figure 1. Seasonal variation in the moulting rhythm of groups of *B. balanoides* L. collected at approximately fortnightly intervals during the years 1954–57. Periods when fertilisation is taking place are shown by dark rectangles, and periods when liberation takes place are shown by clear rectangles.

B. balanoides L., *B. perforatus* Bruguière and *Chthamalus stellatus* Poli, but it is difficult to say to which species these exuviae belonged. Darwin also quotes Mr. Thomson's observation that 20 specimens of *B. balanoides* kept alive for 12 days shed 21 casts; presumably one must have moulted twice and the rest once. However, no information is given regarding the condition under which they were maintained.

The seasonal differences in the frequency of moulting of *B. balanoides* over a period of two successive years were determined from the moulting frequency of groups collected at short intervals, roughly once a fortnight, after which a new batch was substituted for the previous batch. It was found unnecessary to feed this species artificially, since for these short periods the moulting rate was not

appreciably influenced (see below and Table III). The moulting rates of such groups are presented in Figure 1 as per cent casts per day, together with temperatures both of the sea and of the cool basement where the groups were kept for observation.

It will be clearly seen from Figure 1 that the rate of moulting, which was about 8–12% per day in September–October, fell dramatically by November–December after the natural population had become fertilised. During December–January animals almost ceased to moult. The period of anecdysis before the animals resumed moulting varied slightly with the individual and its environment. Thereafter the moulting rate increased gradually from 3–5% per day during late February–March, to a maximum value of 8–12% per day by late April–May. However, it dropped slightly by June–August, but increased thereafter till breeding activity started.

The seasonal variation of temperature did not correspond in any way with changes in the frequency of moulting, for, though the temperature remained fairly high during summer, the frequency of moulting in both years diminished in midsummer and rose again by September and October.

Loss of Penis

Crisp and Patel (1958) reported that in *B. balanoides* only, the first cast after the period of anecdysis contained all tissues of the penis, separated by an abscission layer of new cuticle, and that this phenomenon took place whether the particular animal had been fertilised or not. Figure 2 shows the appearance of the cast skin of this species at different times of the year and the opaque appearance of the penis (Fig. 2b) in the first cast after the end of anecdysis is due to the tissues remaining in it.

During the year 1957–58 measurements of the size of the penes of from 10 to 15 cast skins were taken every day. The individuals were of the same approximate age group, mostly exceeding two years; no young individuals were used. During the period of anecdysis, measurements of penis length were made from individuals removed from the experiment. The results are shown as a series of mean values in Figure 3. A new penis gradually developed during the period of summer growth, reaching its maximum length before the onset of the next breeding season (Fig. 2a), and was subsequently lost after the period of anecdysis as described above.

Figure 4 illustrates the moulting behaviour after fertilisation had occurred and shows that all individuals gave out this particular type of cast skin once, and once only, after anecdysis; subsequent moults were normal, a stump being left in the position of the penis (Fig. 2c).

In order to confirm that the animals did not give off their penes as a result of laboratory conditions, a number of specimens were collected from the shore during the second week of January, 1957, and the penis length measured. Figure 5 shows the distribution of penis length throughout the population. The results show a clear bimodality on account of there being two types of individuals, those which had lost and those which had retained the penis. By distinguishing between those which had lost and those which had retained the penis it was possible to

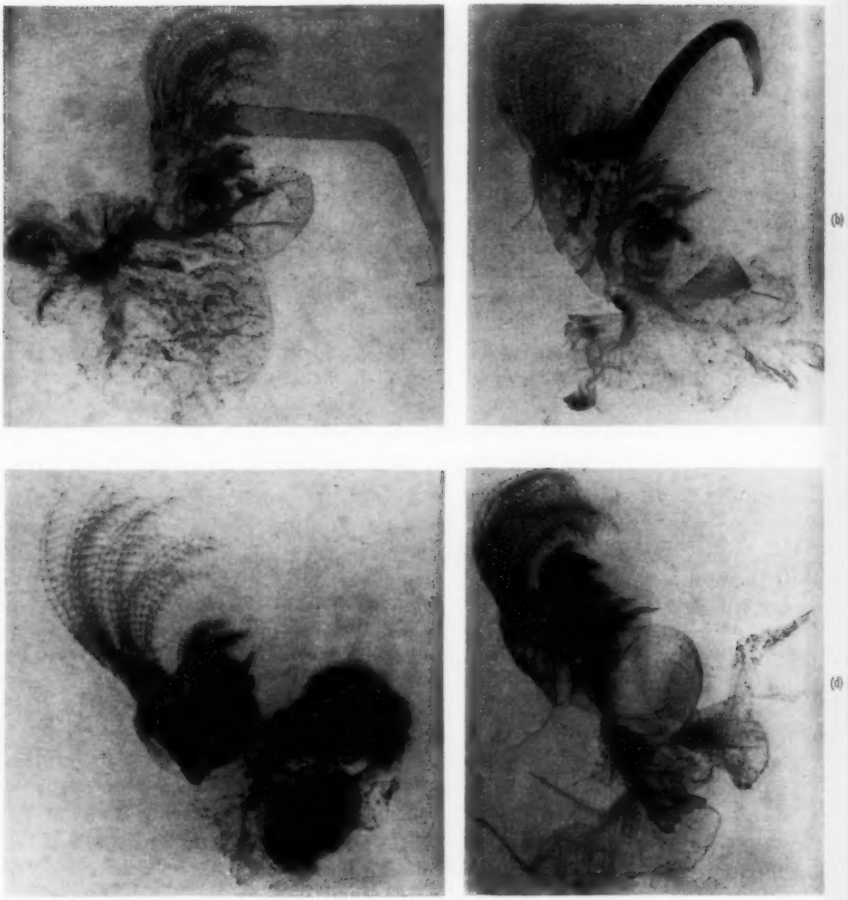

FIGURE 2. Photographs of the cast skins at different times during the year, showing the seasonal changes in appearance of the penis. (a) September–November: Cast with the skin of fully developed penis. (b) January–February: First cast after the period of anecdysis with all tissues of the penis shed in the penis skin, rendering it opaque. (c) March–April: Post liberation cast with remains of egg masses, scarcely any penis present. (d) May–August: Summer cast with developing penis stump.

show that the loss of penes occurred earlier in barnacles growing at high water mark than those growing at mean tide level and low water mark (Table I). This appeared to be due to the fact that breeding activity started first in those settled high up on the shore (Crisp, 1959) and which were therefore correspondingly in advance of those lower down in casting the penis.

To investigate whether fertilisation was a necessary prelude to the loss of the

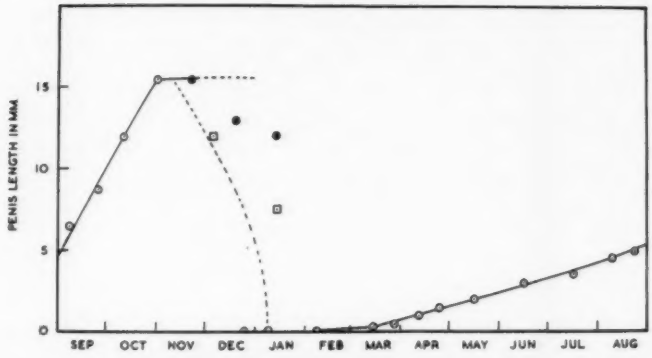

FIGURE 3. Seasonal changes in the development of the penis, measured from the cast skins. ⊡, mean values from a highly bimodal distribution of lengths due to some individuals having shed the penis. The mean size of those retaining the penis is given thus ●. Note the shrinkage in size after fertilisation, and eventual loss of the organ.

penis, mature animals collected at the end of October and early November were isolated in dishes to prevent the occurrence of copulation and fertilisation of eggs. They were maintained at temperatures corresponding closely with those in the sea. Not only the individuals which already had fertilised egg masses, but also

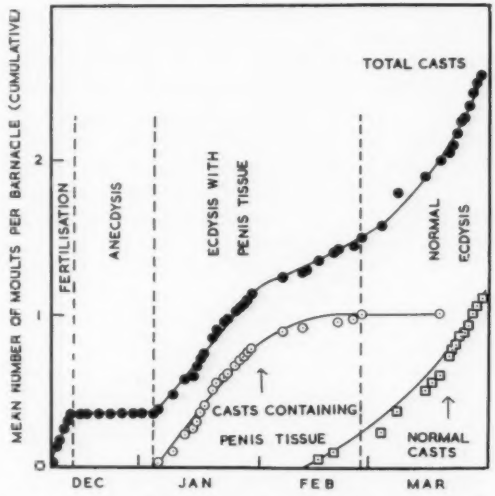

FIGURE 4. Moulting rate of 36 specimens of *B. balanoides* L. at 7° C. between 8.XII.1957 and 29.III.1958 showing how, after the period of anecdysis, the first moult contains all tissues of the penis and the subsequent moults are normal. ●, total number of all casts shed. ⊙, casts containing penis tissue, shed in January and February, one only per barnacle. ⊡, normal casts shed after the middle of February.

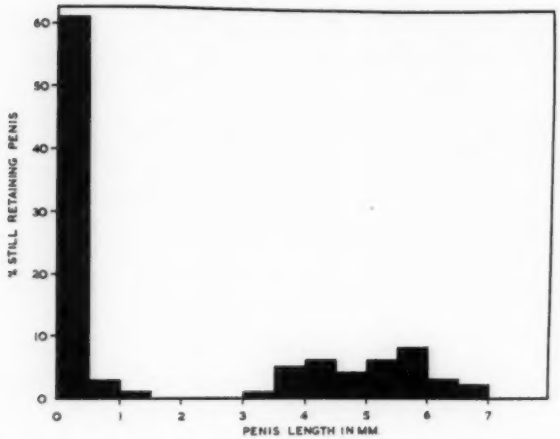

FIGURE 5. Distribution of penis lengths in a natural population of about the same year group on January 11th, 1957, showing clear bimodality on account of there being two types of individuals, those having lost and those still retaining the penis.

those which were still unfertilised gave off the cast with penis tissues (Table II). This indicates that fertilisation of itself had no marked effect on the loss of the penis.

B. balanoides could also be prevented from becoming fertilised by keeping them at fairly high temperatures (15°–21° C.) throughout the summer and autumn. These unfertilised animals continued to retain a normal penis, but if they were subsequently transferred to a temperature of 6° C. and kept cool for several weeks, they entered the breeding condition, became fertilised, and showed the typical pattern of anecdysis and loss of penis. Others were maintained at 5° to 7° C. throughout the summer and autumn, but, on account of abnormal laboratory conditions not fully understood, reached maturity considerably later than those kept in the cool basement or those growing normally in the field. These animals, although kept in the cold for a long period, retained a normal penis until breeding had taken place. After having bred, all individuals gave off their penis with the first cast following anecdysis. Furthermore, in some experiments one set of barnacles were starved and another fed; this did not influence in any way the loss of the penis.

It is clear therefore that the loss of the penis is not directly dependent on fertilisation nor on maintaining the animals at low temperature, nor on the availability of food. It appears to be a part of a normal physiological cycle, in which the gonads as a whole undergo recession, the loss of the penis being accompanied by

TABLE I

Percentage of fertilised population, collected from different tide levels, which had still retained the penis on January 11th, 1957

Level	% with penes
H. W.	10.5
M. T.	20.4
L. W.	37.8

TABLE II

Percentage of animals which lost the penis after the period of anecdysis when maintained at 7° to 12° C.

Date animals were removed for examination	Number of specimens used		Percentage which gave off casts containing penis tissues	
	Animals without egg masses	Animals with egg masses	Animals without egg masses	Animals with egg masses
9.I.1958	188	25	42.5	28.0
11.I.1957	14	12	50.0	50.0
21.II.1958	30	36	97.0	94.0

shrivelling of the vesiculae seminalis and degeneration of testes. This takes place only after they have experienced such conditions as allow the gonads to reach their full development, and under normal circumstances for them to become fertilised.

This phenomenon was observed only in this species. Crisp (1954), however, had reported in *Balanus porcatus* (da Costa) a shortening in the length of the penis after fertilisation, followed by gradual lengthening during the summer, reaching a maximum length just before copulation.

MOULTING RATE OF YOUNG BARNACLES

Costlow and Bookhout (1953, 1956) studied the moulting rate of recently settled cyprids of *Balanus amphitrite niveus* and *B. improvisus* under varying conditions of light and food till they reached the adult stage.

During the present investigation the moulting rate of recently settled spat of *B. balanoides* was studied.

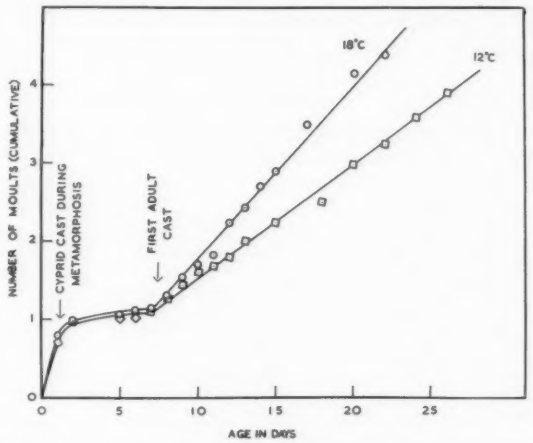

FIGURE 6. Moulting rate of young spat of *B. balanoides* L. of known age when kept at 12° C. and 18° C.

Cyprids which had settled on inert plastic plates and metamorphosed were brought into the laboratory and kept in dishes. The water in the dishes was stirred mechanically and *Chlamydomonas* sp. were offered every day as food. Since satisfactory conditions could not be guaranteed in the laboratory, these plates were returned to the sea. At the same time another batch, which had been put out for settlement, was brought into the laboratory, and was placed in a dish; the water was stirred and the animals fed as before. Several plates were thus kept in rotation between the laboratory and the sea, a few plates being kept continuously in the laboratory. The number of exuviae was recorded each day.

Figure 6, drawn from the sets of results, illustrates the cumulative number of casts shed during the first few weeks after settlement. Metamorphosis of cyprids occurred after 24 to 36 hours and the apodemes of the paired eyes, the bivalve carapace and the exuviae of the thoracic limbs were shed (Crisp and Stubbings, 1957). No further ecdysis occurred for at least 5 days. The time of the first adult cast varied considerably, but on average it took place between 9 and 10 days after metamorphosis. This was longer than the corresponding period for *B. amphitrite* (Costlow and Bookhout, 1956), in which the first cast occurred between 3 and 5 days after metamorphosis. Thereafter *B. balanoides* moulted approximately once every four days at 18° C. and about once every six days at 12° C. From Figure 6 it will be clearly seen that the moulting rate of the rapidly growing spat was at least double the maximum rate of moulting of the adult over the same period of the year (Fig. 1).

Factors Influencing Ecdysis

a. *Feeding*

Groups of *B. balanoides* were maintained at several temperatures, some being fed on *Artemia* larvae and other groups having the water changed only. Those groups which were given *Artemia* were observed to be beating the cirri intermittently throughout the day and producing dark red faeces. The starved groups, on the other hand, were active for a short time after the water in the dishes had

Table III

Moulting rate of B. balanoides under varying conditions of temperature and feeding. The animals were collected on July 1st, 1957

Number of animals available	Condition		Rate of moulting	
	Fed or starved	Mean temperature	First 10 days after collection	First 60 days after collection
46	fed	19.5° C.	0.10 casts per day	0.13 casts per day
47	starved		0.10 casts per day	0.065 casts per day
22	fed	17.0° C.	0.12 casts per day	0.12 casts per day
21	starved		0.10 casts per day	0.06 casts per day
27	fed	6.0° C.	0.05 casts per day	0.046 casts per day
29	starved		0.05 casts per day	0.032 casts per day
27	fed	3.0° C.	0.05 casts per day	0.03 casts per day
27	starved		0.05 casts per day	0.03 casts per day

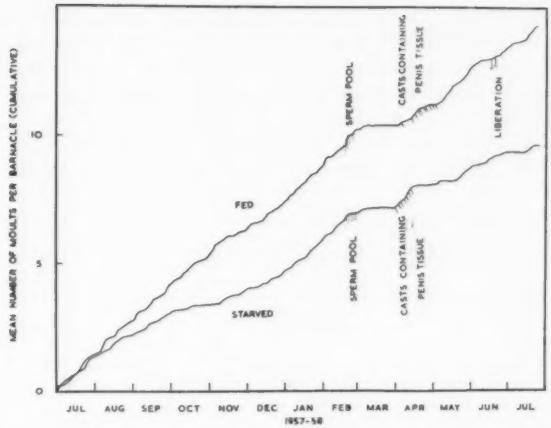

FIGURE 7. Moulting rate of groups of *B. balanoides* L. showing the effect of starvation at 5–7° C. Sperm pools indicate that fertilisation was taking place. Casts containing penis tissues are shown by oblique lines and liberation by small arrows.

been changed, but remained quiet for the rest of the day except for very occasional beating.

As can be seen from Table III, both fed and starved batches moulted at approximately the same frequency immediately after collection and for the following ten days. Thereafter the fed barnacles continued to moult at the same or at a slightly decreased rate, but the starved barnacles showed a progressive reduction in frequency but did not cease to moult altogether. These long-term changes are shown in Figure 7 for animals kept at 5 to 7° C. It will be noticed that after anecdysis the moulting rate was low, but it recovered much more rapidly in those individuals which were fed. These results suggest that the amount of reserve food available is important in determining the moulting rate. The results in Table III show that at higher temperatures there is an even more marked difference between fed and starved individuals due, no doubt, to the greater loss of reserve food at the higher metabolic rate.

b. *Temperature*

Southward (1955a), who studied the influence of temperature on the cirral activity of *B. balanoides*, reported that the rate of cirral beats increased linearly with the increase in temperature from 3 to 20° C. The rate of beating, however, fell with a further rise in temperature, 31 to 32° C. being lethal.

Similarly (Fig. 7) the moulting rate of barnacles fed on *Artemia* increased linearly with the rise in temperature from 3 to 20° C. The moulting rate of starved specimens, on the other hand, increased from 3 only up to 12° C. and then diminished with a further rise in temperature. If, as seems likely, the rate of moulting is influenced by the food reserves available, it would not be surprising that with increased metabolic rate at higher temperatures the reserves would fall

sufficiently to reduce the moulting rate. After a sufficient period the effect of loss of reserves might more than counteract the normal increase in moulting rate with temperatures, so that the moulting rate actually fell with rise in temperature.

Both fed and starved specimens moulted at the same frequency at the lowest temperature (Table III, Fig. 8); the reason for this might be the fact that at 3–4° C. those which were offered food remained quiet and fed very little, while those which were starved did not lose reserve metabolites at any appreciable rate at low temperatures.

Southward kept his specimens only for a very short time at 25 to 30° C. and was able to measure their cirral beat at these temperatures; however, both fed and

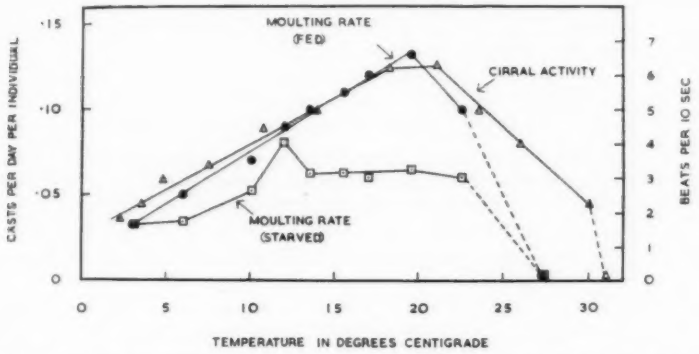

FIGURE 8. The effect of temperature and feeding on the moulting rhythm of *B. balanoides* L. and the effect of temperatures on cirral activity (from Southward, 1955a).

starved specimens during the present series of experiments died if kept for more than three days at 25 to 27° C.

c. *Breeding*

The majority of recently fertilised animals abruptly ceased to moult for some six to eight weeks (Fig. 9); this might be regarded as an adaptation to prevent recently oviposited eggs, which are not yet hardened, from being shed with a cast. However, after anecdysis, the still gravid barnacles resumed moulting, especially if they were fed (see Figure 7) and were found to have cast the skin with a torn-off mantle lining, due to the pressure exerted by the egg masses. The latter had by this time become hard and were pressed well up against the mantle lining of the parietes and basis. Occasionally only the exuviae of the appendages and prosoma were shed, the mantle lining being retained and shed later at the time of the liberation of the nauplii hatched out from the egg masses.

It will be seen from Figure 9 that the barnacles which were kept from being fertilised by being artificially isolated continued to moult when the fertilised population had ceased. Nevertheless they showed a sharp fall in moulting rate by the end of November. This suggests that the drop in the moulting rate may be due to a basic physiological rhythm, like that controlling the loss of the penis and

degeneration of the gonads, and that the sudden onset of this process in fertilised barnacles may be due to the stimulus of copulation or oviposition.

d. *Effect of emersion on moulting*

As Darwin (1851) observed, the skin cannot be shed except when the barnacle is immersed in water. The period of emersion of organisms which grow between low water neap and high water neap tide levels never exceeds about 12 hours. In a humid environment intertidal barnacles may be kept out of water quite healthily

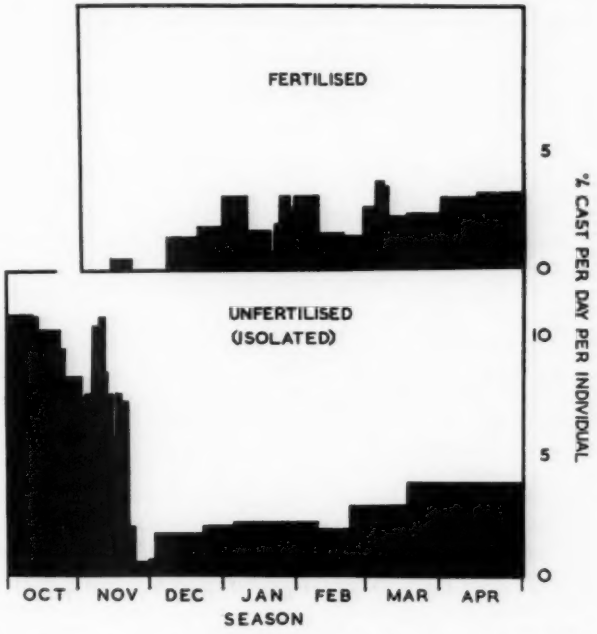

FIGURE 9. Moulting rate of isolated specimens not carrying embryos and of those with fertilised egg masses, measured over the same period. Fertilised specimens show an immediate drop to zero; unfertilised specimens continue to moult freely in mid-November, but show a fall in late November at the end of the normal breeding season.

for several days. It is thus possible to ascertain whether the frequency of moulting is influenced by an abnormally long period of emersion.

Four groups of barnacles growing under nearly identical conditions on settlement plates on a raft were brought into the laboratory. Here they were kept under a damp cloth for varying periods of time. The total number of casts shed when the barnacles were introduced for a period of 24 hours into aerated sea water was recorded. In Table IV the aggregate percentage of casts (per 100 barnacles) is shown against the total number of days kept in the laboratory. The letter "D"

TABLE IV

Moulting rate in relation to the treatment of various groups of barnacles; letter "D" indicates the days when the specimens were kept dry, on the other days they were kept in sea water at 12° C.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Group	Number of barnacles used in experiment	1st day	2nd day	3rd day	4th day	Total casts after 4th day
a	382	D	D	D	26.2%	100
b	373	D	D	22.0%	35.4%	132
c	370	D	14.3%	20.2%	28.6%	106
d	453	3.7%	D	D	33.1%	150
Mean	—	—	—	—	30.9%	(7.7 ∞ per day)

$\chi^2 = 9.98$

indicates the days when the groups were kept dry, and no casts were therefore emitted. Column 7 gives the total number of casts for the whole period.

It is clear that moulting proceeds steadily throughout the experiment and is not delayed significantly as a result of the barnacle being out of water. The numbers of casts found after 4 days in the different groups, a, b, c and d, which were treated differently give a value for χ^2 of 9.98 when compared with the expectation of 7.7% per day per individual. This is slightly higher than the value of $\chi^2 = 7.8$ for the 5.0% significance level. Since, however, there was no consistent trend in the moulting rate in relation to the emersion period, the significance of the χ^2 value was probably due to intrinsic variations in the rate of casting of the four groups, a, b, c and d; such variations are frequently observed between groups of individuals from apparently similar habitats. The data of Table IV, rearranged to show the moulting rate in relation to the time kept out of water, are displayed in Table V. The rates of casting are closely uniform and the χ^2 test applied to the differences was found not to be significant ($\chi^2 = 6.26$ for 3 degrees of freedom). The number of casts produced in a given time is therefore not altered significantly by emersion up to a period of four days.

TABLE V

Moulting rate in relation to the total time out of water

(1)	(2)	(3)	(4)
Number of days out of water	Barnacle days (No. of barnacles × no. of days)	Number of casts	Moulting rate (% casts per day)
4	1528	100	6.55%
3	2478	215	8.69%
2	740	53	7.17%
1	1566	120	7.68%

$\chi^2 = 6.26$

It was often noticed that the rate of moulting for the first day or two was higher than the average for the whole period. This may have been partly due to the individuals having been out of water since the previous high tide. Possibly also the changed conditions in the laboratory caused a temporary rise in the rate of moulting. However, this small initial rise had no significant influence on the average rate taken over two or three weeks.

e. *Tidal periodicity*

In many marine organisms, especially in lamellibranchs and in annelids, the effect of moon and tide on the breeding cycle had been well established (Korringa, 1947.) However, Crisp and Davies (1955) reported that *Elminius modestus* bred at any time irrespective of the tidal cycle.

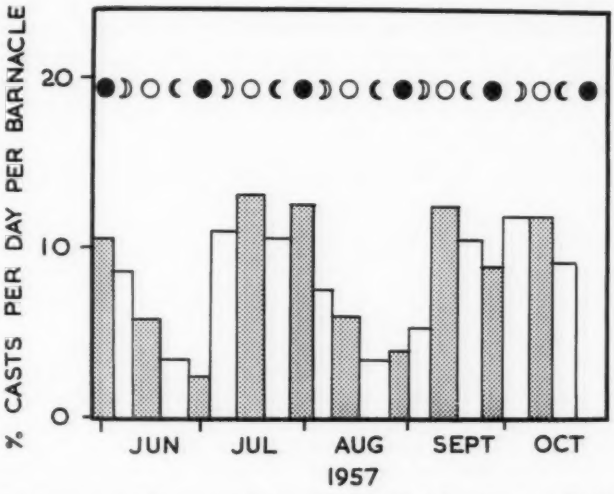

FIGURE 10. Average moulting rate of the groups of *B. balanoides* L. collected at monthly intervals and kept at sea temperature over the spring and neap tide periods (shown by moon phases). Stippled blocks, average moulting rate over the spring tide period; clear blocks, average moulting rate over the neap tide periods.

Churchill (1917–18) studied the effect of the lunar cycle on the moulting rhythm of the blue crab *Callinectes sapidus,* but on the basis of his results dismissed the popular belief prevailing that both moon and tide had a marked effect on the moulting of crabs as being a folklore superstition. However, Wheeler (1937) in *Anchistioides,* Nouvel and Nouvel (1939) in the mysid *Praunus flexuosus* and Nouvel (1945) in *Leander serratus* found that the largest numbers of exuviae were shed on the day corresponding to the largest tide. However, their observations were made only over a single lunar period and may therefore be fortuitous. Kinne (1953, 1959) found no tidal rhythm in the moulting of the amphipod *Gammarus duebeni.* Tidal rhythm in moulting does not therefore seem to be of general occurrence in the Crustacea.

During the present investigation experiments were carried out to test if any tidal rhythm could be found in barnacles. Each tidal cycle was divided into two periods, approximately midway between the largest and the smallest tides, one period corresponding to the spring and the other to the neap tides (shown by moon phases in Figure 10). The average moulting rate of groups of animals collected at monthly intervals was determined for pairs of successive neap and spring periods. The difference between the average for neap and spring periods was tested by applying Student's "t" test and found to be insignificant. Ecdysis therefore occurred at approximately regular intervals, provided barnacles were immersed, irrespective of the tidal cycle.

f. *Effect of tidal level*

Animals were collected from three different tidal levels and their moulting rate for the first ten days was recorded. It can be seen from Table VI that there was no significant difference in the moulting rhythm. Though Southward (1955b) did record specimens (*B. balanoides*) collected from low water mark beating

TABLE VI

Moulting rate of B. balanoides, *collected from different tide levels, for first 10 days*

Dates animals were collected	Temperature	Casts per day per individual		
		H. W.	M. T.	L. W.
22nd Jan.	8–10° C.	0.03	0.04	0.05
29th Jan.	8–10° C.	0.025	0.03	0.04
28th Feb.	8–10° C.	0.06	0.04	0.04
5th Sept.	14–16° C.	0.110	0.10	0.12

markedly faster than those collected from higher up, when he repeated the experiment on the following day with the same specimens he found that the differences had disappeared. Neither moulting nor cirral activity appears to be influenced by tidal level.

g. *Influence of light on moulting rhythm*

It has been claimed that light has an inhibitory action on the moulting rhythm of Crustacea (Nouvel, 1945). However, Costlow and Bookhout (1956) did not find any significant differences in the moulting rate of the series of young spat of *B. amphitrite niveus* reared under different conditions of illumination.

During the present series of experiments the influence of light on the moulting rhythm of adult barnacles was investigated. Groups of barnacles were collected from the shore and maintained under three different conditions. One batch was kept under a light-tight box kept in continuous darkness. A second batch was kept in a continuously illuminated, well ventilated box, illuminated by a 25-watt bulb. The third batch was exposed to natural variations of light conditions, 12 hours light during the day and 12 hours darkness at night. All were kept at the

same temperature of 10° C. No significant difference in moulting rate was found over a period of 50–60 days.

h. *Influence of parasite Hemioniscus balani*

The majority of the species of British barnacles was found to be capable of harbouring the isopod *Hemioniscus balani* in the mantle cavity, in the space normally occupied by the fertilised egg masses. Crisp (1954) found this parasite causing castration of the female gonads in *B. porcatus* (da Costa) and later a similar effect was reported in *Elminius modestus* (Crisp and Davies, 1955).

The effect of the parasite on the moulting rhythm was therefore investigated using infected *B. balanoides, Elminius modestus, Balanus perforatus* and *B. amphitrite* var. *denticulata*. It will be seen from Table VII that the presence of the parasite had no significant effect on the moulting cycle of the host; the intermoult periods of infected and uninfected barnacles were not significantly different. However, as with fertilised individuals which had eggs in the mantle space, the speci-

TABLE VII

Frequency of moulting of specimens infected with Hemioniscus balani *and of uninfected specimens collected from the same locality*

Species	Temperature	Number of animals used in experiment	Percentage infected with *Hemioniscus balani*	Mean intermoult period in days of	
				Individuals with *Hemioniscus*	Individuals not infected
Balanus balanoides	12° ± 2° C.	35	57%	10.9	9.0
Elminius modestus	12° ± 2° C.	10	50%	7.3	7.3
Balanus perforatus	13° ± 2° C.	20	5%	6.0	6.4
Balanus amphitrite	15° ± 2° C.	100	6%	10.3	9.8

mens harbouring the parasite were found to shed exuviae with torn-off mantle linings. On no occasion was the parasite rejected with the cast, except in the large barnacle *B. perforatus*. This species was observed to reject the parasite enclosed within the casts, and this may account for the normally very low degree of parasitism present compared with that found in other species in the same vicinity.

SUMMARY

1. The seasonal variations in the frequency of moulting of boreo-arctic species of the operculate barnacle *Balanus balanoides* L. were studied during the years 1954–57 by observing groups of barnacles kept in the laboratory at temperatures corresponding to that of sea water. The rate of moulting, which was about 8 to 12% casts per day per barnacle during October–November, fell sharply by November–December and fertilised animals underwent a period of anecdysis for about 6 to 8 weeks. They resumed moulting at a slower rate, reaching a maximum by May with a slight decrease in June–August, rising again towards the breeding season in November.

2. In this species the first cast after the period of anecdysis always contained all tissues of the penis, separated by an abscission layer of new cuticle. Neither food, temperature nor the act of fertilisation were directly responsible for the loss of the penis. The evidence indicates that the loss of the penis is part of a physiological cycle in which gonads undergo recession after they have reached full development. A new penis gradually developed during the period of summer growth, reaching its maximum length before the onset of the breeding season.

3. Feeding influenced the moulting rhythm, but the effect made itself felt only after the first 10 to 15 days following collection from the shore. The moulting rate of specimens maintained without food for a period longer than this (*i.e.*, for about 30 to 60 days) fell considerably from the initial value, but animals fed on *Artemia* larvae continued to moult at about the same rate as when freshly brought in from the shore.

4. The moulting rate of specimens given food increased linearly with the rise in temperature from 3–20° C.; on the other hand the moulting rate of starved specimens only increased from 3–12° C. and fell considerably with further rise. Temperatures higher than 25° C. were lethal to both groups.

5. Neither the lunar cycle nor the tidal level had any influence on the moulting rhythm.

6. The parasite *Hemioniscus balani*, which caused castration of the female gonads, did not influence the frequency of moulting.

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EFFECTS OF TURBIDITY-PRODUCING MATERIALS IN SEA WATER ON EGGS AND LARVAE OF THE CLAM (VENUS (MERCENARIA) MERCENARIA)

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The effect of suspended materials on mollusks and on the survival and growth of their larvae is of interest to biologists and of considerable importance to shellfish producers. Since several of the most important commercial species are essentially inhabitants of the shallow waters of bays and estuaries, almost all of the grounds used for the cultivation of shellfish are frequently subject to water rendered turbid by natural phenomena, such as floods and storms, and by human operations, such as dredging, bridge- and road-building, etc.

An extensive field study by Lunz (1938) during the dredging of the Intracoastal Waterway of South Carolina showed that the mortality of adult oysters in the dredged area, except where oysters were actually buried by the spoil, was no higher than in areas remote from the dredging operations. Moreover, he found no evidence that the physiological condition of the oysters, as judged by the yield of meats per bushel of oysters, was affected by the dredging operations. He also reported that the intensity of setting of oysters in an area adjacent to dredging operations did not differ from setting intensity in areas remote from such operations, and concluded (p. 134) that "dredging operations apparently had no effect on spawning and setting."

In careful quantitative experiments Loosanoff and Tommers (1948) have shown that as little as 0.1 gram per liter of silt reduced the average pumping rate of adult oysters by 57 per cent. At a concentration of 1.0 g./l. they found the reduction in average pumping rate was more than 80 per cent and reached 94 per cent when the concentrations of silt were increased to 3.0 and 4.0 g./l. They reported that results with kaolin and chalk were similar to those obtained with silt, and that 0.5 g./l. of Fuller's earth, the only concentration tested, reduced the rate of pumping by 60 per cent.

Since the development of techniques for rearing bivalve larvae in the laboratory (Loosanoff and Davis, 1950), these techniques have been used to determine quantitatively the effects of various factors, such as temperature, species of food organisms, crowding, quantity of foods and salinity on the survival and growth of bivalve larvae (Loosanoff, Miller and Smith, 1951; Loosanoff and Davis, 1953; Loosanoff, Davis and Chanley, 1953a; 1953b; Davis, 1953; Davis and Guillard, 1958; Davis, 1958). In the present study they have been employed to determine quantitatively the effect of various concentrations of several materials suspended in sea water on the development of eggs of the hard clam, *Venus* (*Mercenaria*) *mercenaria*, and on the survival and growth of their larvae.

The turbidity-producing materials used in these experiments were clay (kaolin N.F. VII Mallinckrodt), Fuller's earth (dusting powder, McKesson), chalk (pre-

cipitated chalk U.S.P. McKesson and Robbins) and silt. The silt was collected from the bed of the Wepawaug River, just upstream from our laboratory. The silt was washed through a 325-mesh, stainless steel screen (meshes approximately 50 microns square) to remove larger particles, collected in a Büchner funnel, where it was washed with distilled water to remove salt, and then dried at 200.0° C. Finally, the dried cake of silt was ground in a jar mill. In the later experiments the Fuller's earth was also ground in the jar mill. Concentrations of suspended material are, therefore, all expressed as grams of dry powder per liter.

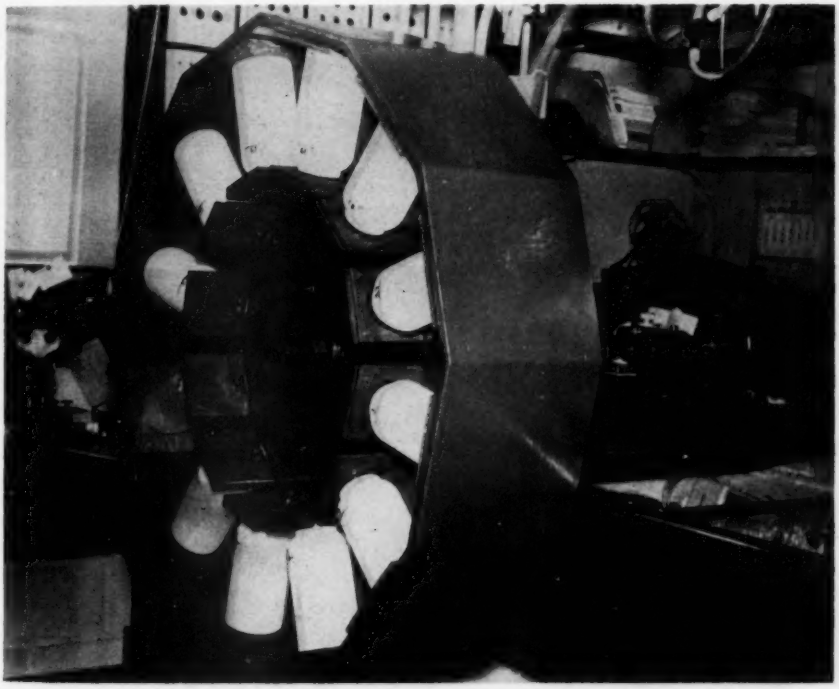

FIGURE 1. Turbidity apparatus showing the wheel and method of attachment of bottles. Motor and reduction gear box indistinctly seen in background.

In making up a suspension, a weighed quantity of the powdered material was thoroughly mixed with sea water and the various experimental concentrations prepared by serial dilution.

The method for obtaining fertilized clam eggs in midwinter has previously been described (Loosanoff and Davis, 1950) as have the methods for determining the percentage of eggs developing to the straight hinge stage, and for determining the rate of growth of larvae (Davis, 1953, 1958).

In the present series of experiments, wide-mouthed, 32-ounce polyethylene bottles with screw caps were used as containers for the larval cultures. To keep

the materials in suspension we used a modification of an apparatus which V. L. Loosanoff of this laboratory observed in P. R. Walne's laboratory at the Fisheries Experiment Station, Conway, England. The bottles were mounted on a vertical wheel which, as it rotated, turned the bottles end over end once for each revolution of the wheel. In our apparatus provision is made for 12 bottles on each side of the wheel for a total of 24 bottles so that 12 sets of duplicate cultures can be run concurrently (Fig. 1). The wheel is kept rotating by a constant speed motor working through a speed reducer giving eight revolutions of the wheel per minute. The wheel and attached bottles are enclosed in a plywood box in which the air

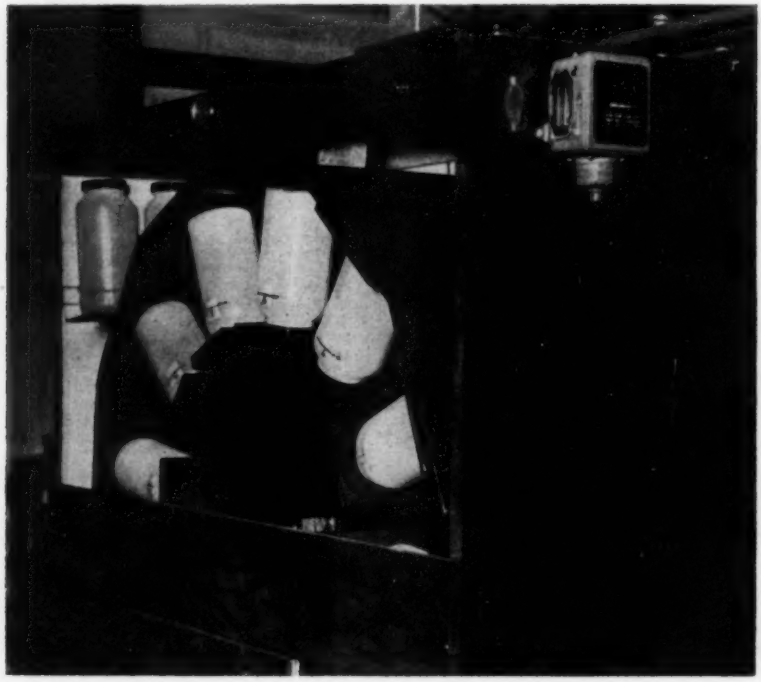

FIGURE 2. Turbidity apparatus showing thermostat control, temperature box and position of stationary control cultures.

temperature is thermostatically controlled at 24.0° C., using two 60-watt light bulbs as heaters. A wire mesh shelf is installed in the left upper corner of this box to hold two additional bottles for stationary control cultures (Fig. 2). In practice, one pair of bottles on the wheel is used for moving control cultures, leaving 11 pairs of bottles for duplicate cultures of larvae at each of 11 different sets of experimental concentrations of suspended materials.

The number of larvae suspended in the 800 ml. of sea water and turbidity-producing material in each bottle was initially the same for all cultures in an

experiment, but ranged from 8000 per bottle in some experiments to about 15,000 in others. The larvae were fed daily, each culture receiving an equal quantity of a mixture of *Isochrysis* and *Monochrysis*. The larvae were examined every second day when the sea water and its suspended material were changed, but quantitative samples for counts and measurements were taken only when the larvae were 48 hours old and on the twelfth day, when an experiment was terminated.

Preliminary experiments indicated that some fertilized clam eggs could develop into straight hinge larvae in concentrations of either clay (kaolin) or chalk as high as 4.0 g./l., but that none developed to the straight hinge stage in equivalent concentrations of either Fuller's earth or silt. Subsequent experiments, however,

TABLE I

Percentage of clam eggs developing to the straight hinge larval stage in different concentrations of suspended materials. The number of eggs developing to the straight hinge stage in the stationary controls is considered 100 per cent

Concentration g./l.	Percentages			
	Silt	Clay (kaolin)	Fuller's earth	Chalk
Stationary control	100	100	100	100
Moving control	91			
0.125	95	82	75	
0.188	90			
0.250	96	82	61	approx. 45
0.375	93			
0.500	99	52	41	
0.750	92			
1.000	79	37	57	approx. 30
1.500	65			
2.000	39*	49	50	approx. 39
3.000	0			
4.000	0	42**	45***	approx. 45****

* Averaged 195.80 μ
** Averaged 203.90 μ
*** Averaged 199.60 μ
**** Averaged 189.10 μ

At 12 days when kept in these turbidities only during the first 48 hours after fertilization and then returned to normal sea water.

have shown that if Fuller's earth was first ground in a jar mill, some larvae developed normally at 4.0 g./l. Similarly, ground silt at concentrations of 3.0 or 4.0 g./l. still completely prevented normal development of clam eggs (Table I). In silt concentrations of 0.75 g./l. or lower, however, there were no significant differences in the percentage of clam eggs developing normally, while with clay, chalk or Fuller's earth there appeared to be a more or less consistent stepwise reduction in the percentage of eggs developing normally with each increase in the quantity of suspended material (Table I).

Although in a silt concentration of 2.0 g./l. only 39 per cent of the eggs developed to the straight hinge larval stage, almost all of these larvae were capable of surviving and growing to metamorphosis (mean length 195 μ at 12 days) if

returned to normal sea water. Similarly, larvae developing to straight hinge stage in concentrations of 4.0 g./l. of clay, chalk or Fuller's earth have been reared to setting stage after being returned to normal sea water at the end of 48 hours.

Experiments on the effect of suspended materials on the growth of clam larvae that have developed to the straight hinge stage in our usual sea water have shown that such larvae cannot grow in concentrations of clay, Fuller's earth or chalk as high as those at which some eggs developed. For example, there was no evi-

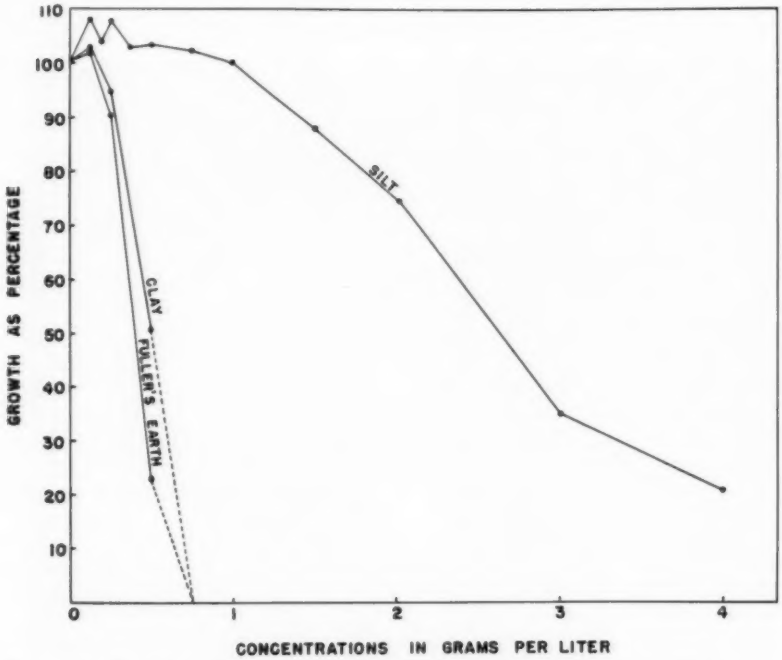

FIGURE 3. The 12-day increase in mean length of clam larvae, grown in different concentrations of suspended materials, plotted as percentages of the increase in mean length of larvae in control cultures. Each point represents the average for 50 larvae from each of duplicate cultures in each of two experiments.

dence that the larvae were taking the flagellates provided as food, and there was no growth of the larvae in a concentration of chalk as low as 0.250 g./l. With either clay or Fuller's earth, 0.500 g./l. was the highest concentration in which the larvae showed evidence of taking food (color in digestive gland) or any increase in size (Fig. 3). In cultures receiving higher concentrations of clay or Fuller's earth, many larvae ingested some of the suspended particles, apparently in sufficient quantity to block the digestive tract and cause death. Kaolin in a concentration of 0.500 g./l. caused approximately 50 per cent mortality in 12

days and almost complete mortality at all higher concentrations. There was no appreciable mortality of larvae in 0.500 g./l. of Fuller's earth in 12 days, but at all higher concentrations mortality exceeded 90 per cent.

When silt was used as the suspended material the results were quite different from those with clay, Fuller's earth or chalk. Clam larvae showed evidence of taking food (color in digestive gland) even at a concentration of 4.0 g./l. of silt, but growth was negligible, and it is doubtful if any larvae in natural waters could reach metamorphosis in either 3.0 or 4.0 g./l. of silt. Growth of most larvae was also considerably retarded by concentrations of 2.0 g./l. or even 1.5 g./l. of silt, but some larvae under these conditions had reached setting size (maximum length 185 μ and 200 μ, respectively) by the twelfth day. Growth of clam larvae was approximately normal in 0.750 g./l. of silt and, at all lower concentrations, was somewhat better than that of larvae in the control cultures (Fig. 3). There was no appreciable mortality of clam larvae, within 12 days, in any of the concentrations of silt tested.

Soil particles ranging in size from 62 microns to 4 microns are listed as silts, while particles ranging in size from 4 microns to 0.24 microns are listed as clays. From our experiments it would appear that it is the larger particles (coarse silt 62 to 31 microns) in the silt and unground Fuller's earth, or aggregates of particles in other substances that interfered with development of clam eggs, while the smaller particles, characteristic of the clays, probably had little effect on egg development. The results of experiments on growth of larvae, however, seem to indicate that the larger particles, characteristic of coarse silts, may have little effect on growth. It seems to be the smaller particles, as in clays, precipitated chalk, and finely ground Fuller's earth, which are about the size of food cells, that interfere most with growth of larvae. This interference appears to be primarily mechanical through blockage of the digestive tract, but there is some indication that it may also have been, at least in part, an indirect result of an adverse effect of the suspended materials on the food organisms.

The more rapid growth of clam larvae noted in lower concentrations of silt and at the lowest concentrations of clay and Fuller's earth is perhaps due, in part, to chelation of toxic substances, and with silt, in part, to positive growth factors. An improvement in the rate of growth of algal cultures upon addition of soil extract has long been known to botanists, and soil extract has been a basic ingredient in many of the media for algal cultures.

The author wishes to express his appreciation of the suggestions and constructive criticism given by Dr. V. L. Loosanoff, Director of Milford Laboratory. Thanks are also due the mechanical staff who constructed the wheel and constant temperature box, to Kenneth Spencer for preparing the figures and to Miss Rita Riccio for her careful editing of the manuscript.

Summary

1. Some clam eggs developed normally in concentrations of 4.0 g./l. of clay, precipitated chalk or finely ground Fuller's earth, although the percentage developing normally decreased as the concentration of these suspended materials increased.

2. In silt concentrations below 0.75 g./l. the percentage of clam eggs developing normally was not significantly different from that in control cultures but decreased progressively in successively higher concentrations.

3. None of the clam eggs developed normally in silt concentrations of 3.0 or 4.0 g./l.

4. Larvae resulting from clam eggs developing in high concentrations of each of the suspended materials were reared to metamorphosis after being returned to normal sea water at 48 hours.

5. Clam larvae were unable to grow in concentrations of clay, chalk or Fuller's earth as high as those at which some eggs developed.

6. The highest concentration of chalk was 0.25 g./l. and 0.5 g./l. was the highest concentration of clay and Fuller's earth at which clam larvae showed any growth and mortality exceeded 90 per cent at all higher concentrations.

7. In a silt concentration of 0.75 g./l. growth of clam larvae was approximately normal and at lower concentrations was slightly faster than that of larvae in control cultures.

8. In silt concentrations of 1.0 to 2.0 g./l. growth of clam larvae was retarded and at 3.0 and 4.0 g./l. growth was negligible.

9. Even at a silt concentration of 4.0 g./l. there was no appreciable mortality of clam larvae within 12 days.

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CATION REGULATION AND SURVIVAL OF THE RED ALGA, PORPHYRA PERFORATA, IN DILUTED AND CONCENTRATED SEA WATER [1]

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Scott and Hayward (1953, 1954), Biebl (1956), and one of us (Eppley, 1958a, 1958b) have considered that a significant feature of ion transport in algae may lie in maintaining a relatively constant intracellular ionic environment, *i.e.*, cellular homeostasis. That transport mechanisms are of survival value is attested to by the great salinity tolerances established for intertidal algae (Biebl, 1952, 1953, 1956, 1958). Although there is increasing interest in homeostatic mechanisms in marine organisms (*e.g.*, Bullock, 1958), data on ion regulation in algae during osmotic stress are scant.

Blinks (1951) has reported the accumulation of K by *Valonia* from slowly concentrating sea water. *Ulva lactuca* loses some K in 70 per cent sea water which is reaccumulated when external Na is returned to normal (Scott and Hayward, 1955).

Ion transport has been implicated in a number of algae in addition to those mentioned. Bergquist (1958a) has studied Na and K movements after desiccation in *Homosira banksii*, MacRobbie and Dainty measured K and Na fluxes in *Rhodymenia palmata* (MacRobbie and Dainty, 1958a) and in the brackish water *Nitellopsis* (MacRobbie and Dainty, 1958b). In each case a high cytoplasmic K content and an Na content somewhat lower than that of the medium are to be noted.

To a degree, salinity tolerances of algae correlate with their intertidal zonation (Biebl, 1952). However, differences are less marked among intertidal and deeper water forms. Obviously other factors are also involved in intertidal zonation and Doty (1946) contends that the duration of exposure, *i.e.*, submergence or emergence, is of prime importance.

Doty and Archer (1950) further suggest that bright sun, rain, and freezing weather are, in that order, the most critical factors in intertidal algal survival. Kanwisher (1957) has recently reported the effects of freezing and drying on the respiration of some intertidal algae. The present paper reports a study of cellular cation concentrations in *Porphyra perforata*, a red intertidal alga, in different sea water concentrations and the role of ion transport in response to osmotic stress is discussed.

METHODS

Tissue volumes. Tissue water was taken as the difference between fresh weight (after blotting twice with tissue paper) and dry weight (24 hours at

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105° C.), divided by the fresh weight. Apparent free space (AFS) (Briggs and Robertson, 1957) was obtained by allowing $S^{35}O_4$ to diffuse into the algal tissues from sea water for two minutes, blotting twice, then placing the tissues in 10 ml. distilled water for 10 minutes. Aliquots of the distilled water containing the labelled sulfate were then counted with gas flow apparatus. The results are expressed as volume per unit fresh weight. Apparent osmotic volume (AOV) (Briggs and Robertson, 1957), corresponding roughly to the microscopically estimated cytoplasmic volume, was taken as the difference between tissue water and AFS for each sea water concentration.

Ion contents. Sodium and potassium contents were determined by flame photometry. Results are expressed in units milli-equivalents per kilogram fresh weight (meq./Kg. FW), or milli-equivalents per liter AOV.

Artificial sea waters. One hundred per cent sea water was prepared according to the following formula: NaCl, 0.55 *M*; $MgSO_4$, 0.027 *M*; $MgCl_2$, 0.027 *M*; $CaCl_2$, 0.01 *M*; KCl, 0.01 *M*. The concentrations were adjusted proportionately for diluted and concentrated sea waters. In some cases diluted sea water was prepared by adding distilled water to natural sea water. Calcium chloride was omitted for Ca-free sea water.

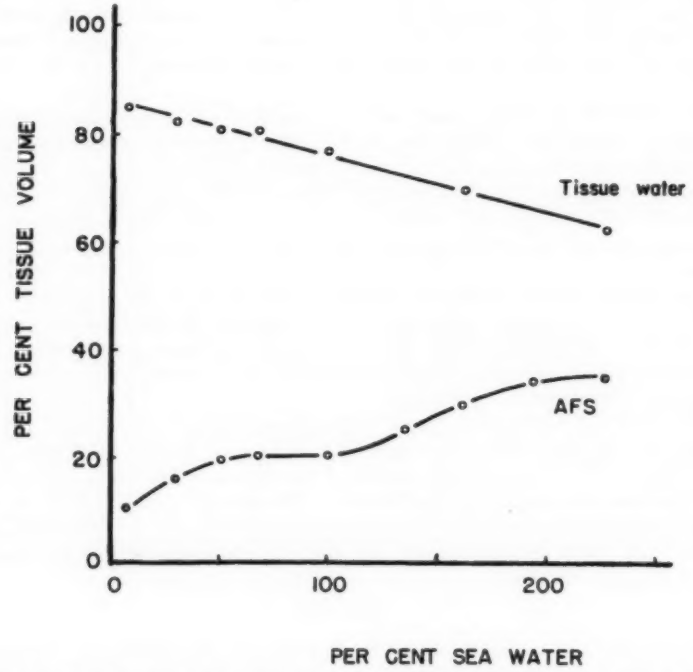

FIGURE 1. Tissue water and apparent free space (AFS) of *Porphyra perforata* as functions of sea water concentration. Apparent osmotic volume is taken as the difference between the two curves.

Results

Tissue volumes at different sea water concentrations. Tissue water and AFS are shown as functions of sea water concentration in Figure 1. AOV is taken as the difference between the two curves. Individual points refer to samples taken

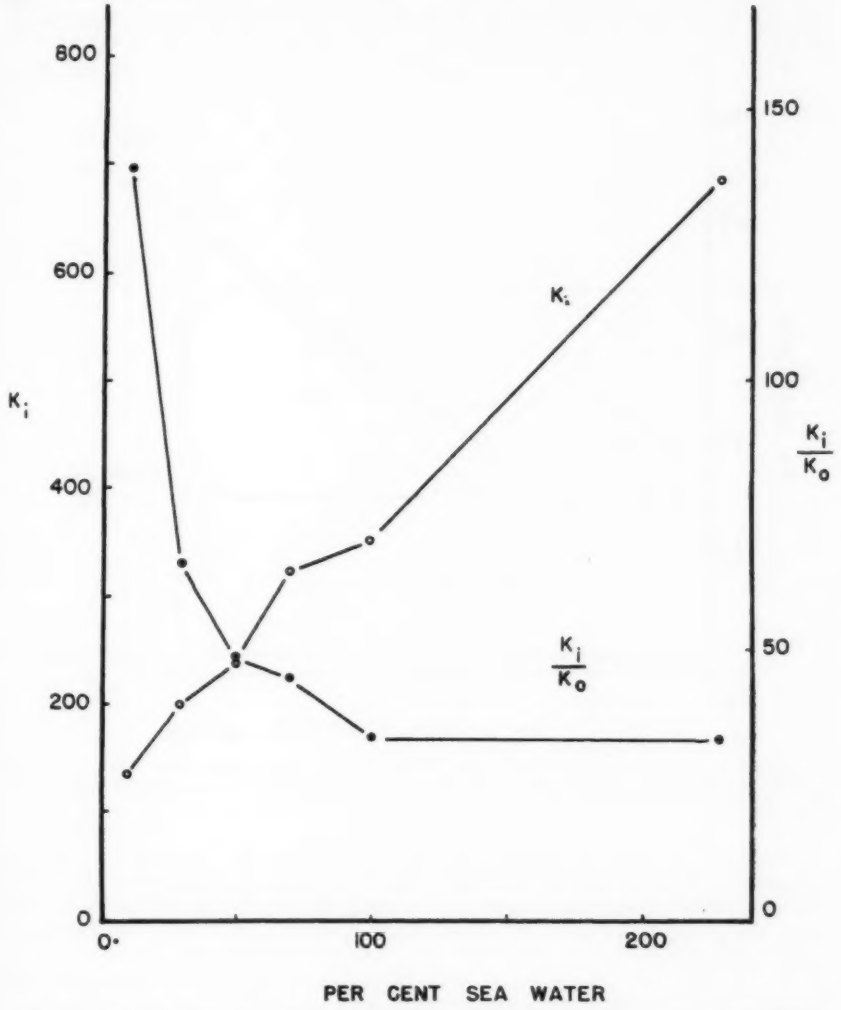

FIGURE 2. Potassium content (K_i), expressed on the basis of apparent osmotic volume (meq./L. AOV), and potassium accumulation ratios (K_iK_o) for *Porphyra perforata* after 24 hours in different sea water concentrations.

after 24-hour exposure. However, samples taken at two minutes give identical results. Volume equilibration takes place very rapidly in the monostromatic algal tissues.

Tissue water follows a linear relationship with concentration. Dry weight provides an increasing component of tissue weight with increasing concentration. Values for weight changes at 50 and 150 per cent sea water are similar to those reported for shrinking and swelling of *Porphyra tenera* blades (Ogata and Takada, 1955).

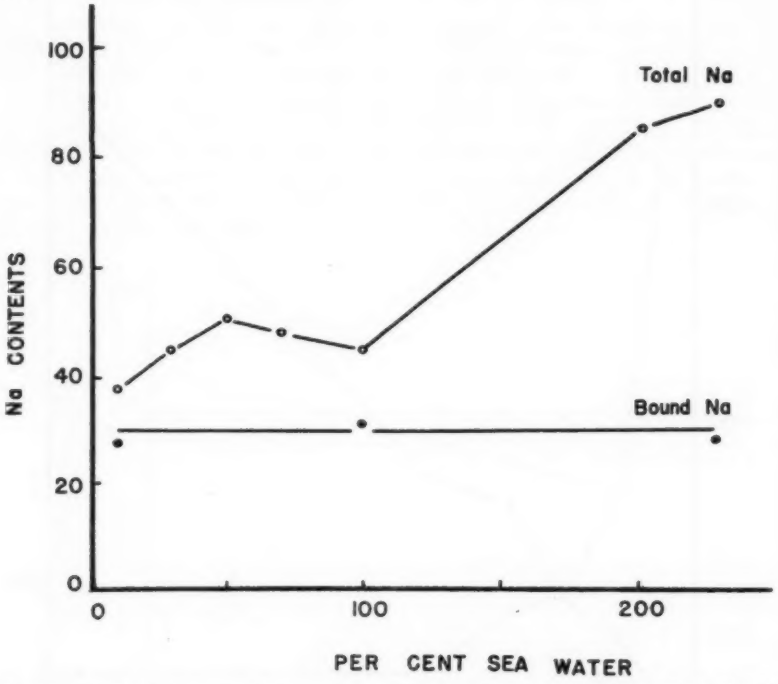

FIGURE 3. Total tissue sodium and bound sodium (the latter determined for killed tissues) in *Porphyra perforata* after 24 hours at different sea water concentrations. Units meq./Kg. FW.

Decrease in AFS in diluted sea water suggests that swelling of the cells squeezes water from the intercellular spaces. At high concentrations the reverse may hold, the extracellular material shrinking disproportionately from the cells. That the intercellular material, a galactan sulfate (Eppley, 1957), is not free to swell indefinitely is indicated by Miwa's study of *Porphyra tenera* (Miwa, 1940). In this species, and probably also in *P. perforata,* at least three structural carbohydrates exist. "Outer membranes," forming a sandwich about the blades, probably restrict swelling. Miwa found these polysaccharide membranes to be hydrolized only with difficulty. The galactan sulfate lies between and immediately

surrounds the cells, each of which has its own discrete wall. None of the three polysaccharides could be identified by Miwa as cellulose.

AOV, taken as an estimate of cytoplasmic volume, shows a relationship to concentration which is reverse to that followed by AFS. Swelling is noted in diluted sea water, a fairly constant volume is maintained between 50 to 100 per cent sea water, and above this value the volume decreases. The cells do not behave as perfect osmometers, nor do they show typical plasmolysis. Both these characteristics are likely due to the extracellular polysaccharides, each of which seems to have distinct swelling and shrinking characteristics. We have no evidence

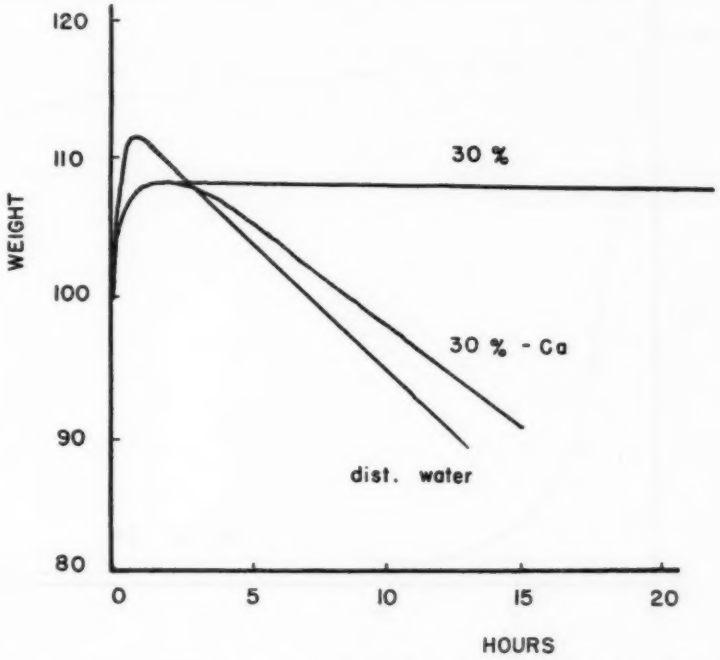

FIGURE 4. Weight, expressed as per cent of the initial weight, of *Porphyra perforata* tissues in 30 per cent sea water with Ca, 30 per cent sea without Ca, and in distilled water. Time course.

that the volume control between 50 and 100 per cent sea water is due to water secretion. Nor does there seem to be any necessity for proposing this as an explanation.

Ion contents in different sea water concentrations. K contents, expressed on an AOV basis, and K_i/K_o accumulation ratios are shown in Figure 2. Cell K follows a linear function of sea water concentration, although a 35-fold, or greater, accumulation is evident throughout. Considerable retention of K occurs in diluted sea waters, resulting in high accumulation ratios, although the actual K content

is lower here than in 100–200 per cent sea water. Little K is bound by the extracellular polysaccharides (1–2 meq./Kg. FW), although large amounts of K may be adsorbed to structural components in other algae, such as *Homosira* (Bergquist, 1958a). Potassium is the principal cellular cation of *Porphyra* at all concentrations studied.

The distribution of Na is less clear, due to appreciable extracellular adsorption. Amounts bound by killed tissues are independent of concentration of sea water for

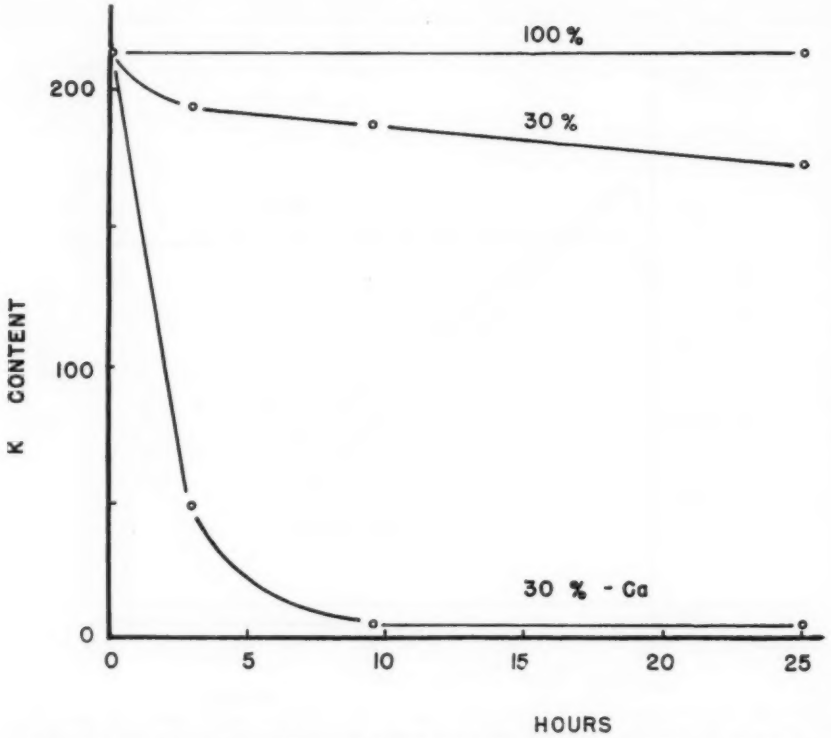

FIGURE 5. Potassium contents (meq./Kg. FW) of *Porphyra perforata* tissues in 100 per cent sea water, 30 per cent sea water with Ca, and 30 per cent sea water without Ca. Time course.

a given plant (Fig. 3), but considerable variation in bound Na is evident in different plants. Therefore Na contents are not expressed on an AOV basis. Throughout the concentration range studied, however, Na is excluded by the cells. The degree of exclusion varies with concentration, but is approximately 10-fold at 100 per cent sea water and increases with increasing sea water concentration.

Total K plus Na thus varies in a roughly linear fashion with salinity, while K is accumulated and Na partially excluded throughout. A similar situation is re-

ported for several halophilic bacteria (Christian and Ingram, 1959). In these bacteria the freezing point depression of cell sap varies directly with sea water concentration, with K contributing significantly to cell osmotic pressure, as does Na.

Calcium and survival in diluted sea water. Tissues placed in 30 per cent sea water without calcium, or in distilled water (Fig. 4) lose weight rapidly after an initial increase. Addition of calcium chloride, 0.01 *M*, prevents the weight loss.

Calcium-free 30 per cent sea water also results in rapid loss of K (Fig. 5), while with Ca the K loss is much depressed. Cell Na, data not shown, increases slightly without Ca (with the concentration gradient), but remains at a lower and constant value with Ca.

TABLE I

Potassium accumulation and sodium extrusion in the presence and absence of $CaCl_2$ or $SrCl_2$ (10 mM/L.). Tissues were first agitated in K-free sea water 20 to 30 hours to render them low in K and high in Na so that subsequent net transport could be measured. They were then transferred to sea water plus KCl.

Expt.	Duration of expt. in hours	K uptake meq./Kg. FW	Na extrusion meq./Kg. FW
1	1.5		
initial		0	0
−Ca		59	26
+Ca		55	38
2	1.5		
initial		0	0
−Ca		46	41
+Ca		60	57
3	19.5		
initial		0	0
−Ca		−7	13
+Ca		96	47
4	16.5		
initial		0	0
−Ca		15	13
+Ca		50	31
+Sr		57	33

Calcium in net transport of K and Na. To investigate the role of Ca in net uptake of K and extrusion of Na, tissues were rendered low in K and high in Na by soaking them in K-free sea water (Eppley, 1958b). They were then placed in sea water with KCl (10 or 20 meq./L.) and the ion contents of the tissues were followed in time. Initial uptake of K and extrusion of Na (Table I) is independent of Ca presence. However, after longer periods net active movements are reduced, indicating loss of K and gain of Na in the absence of Ca. Strontium ($SrCl_2$, 0.01 *M*) may substitute for Ca in preventing loss of K and gain of Na.

Our results are best explained by assuming that Ca-lack gradually brings about leakage through the cell membranes, resulting in increasing movements of salts along their concentration gradients. It seems unlikely that Ca plays any direct

role in the operation of ion transport mechanism, *i.e.*, active transport, but it could well be required to maintain K:Na selective sites on the membrane, or participate in labile membrane structure.

Discussion

Tissue volumes. Because of the lack of a central vacuole and because microscopic estimates of AFS correspond roughly to those measured with $S^{35}O_4$, we have assumed that AFS in *Porphyra* includes the extracellular water but no cytoplasmic component, as is suggested for some higher plant tissues (Briggs and Robertson, 1957). With these assumptions any tissue water not corresponding to AFS must be cellular. AFS has been measured with sucrose for tissues in 100 per cent and diluted sea waters, with results identical to those obtained with radiosulfate. Whether the experimental values include all the extracellular water may be open to question, as well as whether a cytoplasmic component is included. Interestingly, Bergquist (1958b) reported that KCN increases AFS values in *Homosira.* Whether this represents an actual increase in AFS or modification of anionic binding sites was left an open question.

It is with the above reservations that we have taken AOV as the difference between tissue water and AFS, and have calculated K concentrations on this basis.

We do not necessarily presume a uniform cytoplasmic distribution of K, although we plan to investigate this point further. Some localization may occur in vacuomes which are variable in number, but which may comprise a small fraction of cytoplasmic volume. These are visible with neutral red staining. Mitochondria, the chromatophores, and local membrane vesiculations, if they occur, could be sites of variation in cytoplasmic ion concentrations.

While *Porphyra* cells do not behave as perfect osmometers, our results are consistent with the view that active water movements do not take place. Variations in AOV with salinity seem to be due to different degrees of shrinking and swelling of the protoplast and the extracellular, structural polysaccharides.

Ion regulation in concentrated sea water. In corroboration of Biebl's findings (Biebl, 1953), we find that *Porphyra* may survive for some time in 200 per cent sea water. Over the studied range, external ion concentrations of the medium increase equally. Selective precipitation of salts, *i.e.*, $CaSO_4$, occurs only with concentration above 300 per cent. Our investigations have not extended to such concentrations.

Accumulation ratios for K are identical for 100 and 200 per cent sea water. Cell Na shows some increase with concentration, but is lower than that of the medium; thus cation selectivity is retained.

In these experiments changes in salinity were abrupt. Coenocytic algae such as *Valonia* (Blinks, 1951) do not tolerate such rapid changes. Lack of a large central vacuole in *Porphyra* cells may be of importance in this regard. There is no plasmolysis and thus no mechanical injury to the cell membranes with rapid salinity change. AFS and AOV adjustments are almost immediate, as indicated by identical AFS values at two minutes and 24 hours of exposure to concentrated sea water.

Porphyra perforata grows between the 3- and 3.5-foot tide levels, referred to San Francisco (Doty, 1946). Here that alga is regularly covered and uncovered

by the tide twice daily. The maximum period of exposure is 6–8 hours. Concentration of sea water between the algal blades during this period seems unlikely to exceed that tolerated in our experiments, even on the hottest days of summer. Thus we feel that osmotic stress under these conditions is insufficient to result in breakdown of ion transport, resulting in death. Survival in concentrated sea water encountered in the field is not a problem, in our view.

A more serious problem to the emerged algae on hot days is heating, and possibly photoxidation. In summer the superficial algal blades are bleached. Underlying blades (the blades overlap one another) may survive.

Transport and survival in diluted sea water. A 6–8-hour exposure to rain could result in serious injury or death, as indicated by our experiments. Lack of Ca results in rapid loss of K and of weight, and these losses are speeded during osmotic stress. In 100 per cent sea water, free of Ca, *Porphyra* may survive up to 30 hours, compared with only 6–8 hours in 30 per cent Ca-free sea water.

Lack of potassium (Eppley, 1958b) also may influence survival because of the necessity of K for extrusion of Na. Unpublished results indicate that respiration is inhibited about 60 per cent in the absence of Na. Thus the presence of Ca, K, and Na (and probably also Mg) is required for normal operation of cellular processes, one of which is ion transport.

It is of some interest that even in distilled water *Porphyra* tissues adsorb about 10 mM/Kg. FW of Ca. This amount, probably associated with the extracellular galactan sulfate, is not apparently available to the critical sites involved in maintaining membrane selectivity. The implication of this finding may be of some importance in clarifying the role of trace elements passively adsorbed to extracellular polysaccharides in algae. Thus trace element "accumulation" (Black and Mitchell, 1952), in which the extent of adsorption was not determined, might be re-interpreted in the light of this result, with respect to the survival value of such "accumulation." The likelihood remains, however, that in the presence of exchangeable cations, adsorbed trace elements could be made available to the cell surfaces for real accumulation.

Growth experiments which might clarify this problem have not been possible, due to our failure to obtain growth of *Porphyra* in the laboratory. The recent results of Kanazawa and Kashiwada (1959) on the culture of *Porphyra tenera* cast some hope on our aspirations of doing so, however.

A heavy rain, by washing away the sea water film normally present between and around the blades even during emergence, would almost certainly abolish membrane selectivity, decrease respiration, induce loss of cellular cations, and result in high mortality if the blades were exposed long enough. In southern California *Porphyra perforata* largely disappears in the winter, and rain may be involved in this mortality. Other factors are also involved, however.

At the site studied, approximately two miles north of the Los Angeles-Ventura County boundary in California, mass movements of sand were observed during the winter of 1958–1959. The old *Porphyra* bed was entirely covered and a new bed appeared about 50 yards south in the following spring. A smaller sand movement partially covered the new bed in July, 1959. While the extracellular polysaccharides may act as cushions against moderate wave impact and sand scouring, the plants may be torn loose or shredded in a heavy surf or be buried by sand

movements. Such physical processes may be important in survival and distribution as well as physiological tolerances.

Summary

1. Over a wide range of salinity (10 to 200 per cent sea water) cells of *Porphyra perforata* accumulate K and partially exclude Na. Apparent osmotic volume is nearly constant between 50 and 100 per cent sea water. This imperfect volume control is thought to be due to differential shrinking and swelling of structural polysaccharides and not to active water secretion.

2. Survival of *Porphyra* in diluted and concentrated sea water is discussed with respect to ion transport. Rain is potentially a more serious threat to survival, due in part to breakdown of ion transport, than is concentration of sea water. Calcium seems especially important in maintaining membrane selectivity toward K and Na.

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THE EFFECTS OF THYROXIN AND GROWTH HORMONE ON LIVER POLYPLOIDY [1]

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Polyploidy in the cells of the hepatic parenchyma is known to be influenced by changes in the endocrine state produced by hypophysectomy and growth hormone administration (Di Stefano and Diermeier, 1956; Leuchtenberger, Helweg-Larsen and Murmanis, 1954). We have confirmed the effects of both the former (Geschwind, Alfert and Schooley, 1958) and the latter (see below) on liver polyploidy, but have shown that although the hormonal environment may normally play a role in the development of polyploidy, it is not indispensable to that development (Geschwind, Alfert and Schooley, 1958).

In the course of experiments designed to investigate the hormonal factors necessary to stimulate growth in the genetic dwarf mouse (Cole, Geschwind, and Bern, unpublished experiments), it was found that highly purified bovine growth hormone preparations were far less effective in stimulating body growth than were the crude preparations used for this purpose by Fønss-Bech (1947). Since contamination of the crude preparations with the thyrotropic hormone was strongly suspected, it was decided to investigate the effects of thyroxin (more readily available in pure form than is the thyrotropic hormone), alone and in combination with growth hormone, on body growth and liver polyploidy. The results of the experiments on liver polyploidy in these animals, together with the results of a similar series of experiments in the hypophysectomized rat, are reported below.

Materials and Methods

Male and female dwarf mice, 40 to 65 days of age, were apportioned into four groups, one of which served as a control group. The animals in each of the other groups were injected with either 50 micrograms twice weekly of dl-thyroxin (the dose suggested by Nielsen, 1953), 25 micrograms daily of a bovine growth hormone preparation or both hormones at the aforementioned dose levels. Injections were continued for 21 days.[3]

Male rats of the Long-Evans strain were hypophysectomized at 28 days of age. One week later the animals were apportioned into four groups, one of which served as a control group. The animals in each of the other groups were injected with either 2 micrograms daily of l-thyroxin, 25 micrograms daily of the bovine growth hormone preparation, or both hormones at these respective dose levels. Injections were continued for 22 days.

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All animals were sacrificed on the day after the last injection, and the hepatic left lateral lobes were removed from the anesthetized animals, sliced freehand, and fixed in acetic-alcohol. The procedure followed for the preparation of sections for counting and the actual counting procedures have been previously described (Alfert and Geschwind, 1958). Approximately 1000 parenchymal cells were scored in the sections obtained from each animal. The mean percentage distributions of the various types of hepatic parenchymal cells in each of the experimental groups in both series, along with the mean initial and terminal body weights of the animals in each group are recorded in Tables I and II.

Results and Discussion

In the genetic dwarf mouse, the administration of either growth hormone or thyroxin results in a decrease in the number of hepatic mononucleate diploid cells and an increase in tetraploid and binucleate cells (Table I). Of the two hormones, thyroxin, at the dose administered, appears to be more effective in producing these changes. By far the greatest effect, however, was observed in those animals receiving the combined hormone treatment. Such animals also showed the greatest weight gain during the period of injections.

It was difficult to determine unequivocally the manner by which thyroxin brought about its observed effect, since at least three different interpretations of the data could be advanced. One is that thyroxin enhanced polyploidization directly, either by acting as a specific mitogenic agent or by promoting the need for accelerated liver function. A second interpretation might be that the changes in the liver were simply the result of the administration of a large dose of a compound that had to be conjugated and metabolized by the liver, or that was toxic

Table I

The distribution of hepatic parenchymal cell types in control and experimental dwarf mice

Group	Body weight		Mean percentage distribution of cell types*				
	Initial	Terminal	2n	2n bi	4n	4n bi	8n
	g.	g.					
Control**	6.1	7.1	81.4 (78.2–83.1)†	13.1 (10.5–15.3)	3.9 (1.7– 5.2)	0.1 (0.0–0.2)	0.1 (0.0–0.3)
Thyroxin	6.5	10.0	53.4 (49.0–60.4)	18.1 (12.2–23.7)	24.3 (21.9–26.1)	3.0 (1.4–4.8)	0.1 (0.0–0.2)
Growth hormone	7.1	10.3	69.0 (65.4–70.9)	20.9 (17.0–23.2)	8.1 (4.1–10.3)	0.9 (0.6–1.1)	0.0
Thyroxin + growth hormone	6.2	12.1	31.6 (23.4–37.9)	8.6 (5.6–10.2)	52.8 (45.7–61.7)	4.2 (3.3–5.7)	1.6 (1.0–2.3)

* 2n = diploid; 2n bi = diploid binucleate; 4n = tetraploid; 4n bi = tetraploid binucleate; 8n = octaploid. (In all experiments approximately 1% of the cells could not be classified.)

** Three animals per group.

† Range of individual results.

TABLE II

The distribution of hepatic parenchymal cell types in control and experimental hypophysectomized rats

Group	Body weight‡		Mean percentage distribution of cell types*				
	Initial	Terminal	2n	2n bi	4n	4n bi	8n
	g	g					
Control**	74	82	70.1 (67.3–74.5)†	24.0 (20.6–26.5)	4.8 (4.1–5.8)	0.2 (0.1–0.4)	0.0
Thyroxin	77	93	52.3 (46.6–57.8	24.0 (18.1–26.9)	22.0 (19.4–24.6)	0.7 (0.1–1.1)	0.1 (0.0–0.2)
Growth hormone	74	103	53.2 (50.7–58.1)	24.0 (19.9–29.6)	20.6 (17.3–23.6)	0.8 (0.1–1.2)	0.1 (0.0–0.4)
Thyroxin + growth hormone	72	123	22.1 (19.8–26.3)	9.5 (6.4–14.7)	61.4 (56.0–68.1)	3.9 (2.4–6.2)	1.9 (1.6–2.1)

*, **, † Symbols have the same meaning as in Table I.

to the parenchymal cells in the dosage employed. Finally, it was possible that thyroxin, in its capacity as a hormone, promoted repair of pituitary acidophiles which are normally absent in the dwarf mouse (Ortman, 1956), and by so doing stimulated growth hormone secretion. There is good evidence that thyroxin repairs pituitary acidophile cytology in the thyroidectomized rat (Koneff, Scow, Simpson, Li and Evans, 1949; Contopoulos, Simpson and Koneff, 1958), and concomitantly restores growth activity (Eartley and Leblond, 1954; Contopoulos *et al.*, 1958). Such an effect on the acidophiles could explain the findings of Carrière (1955) that in the thyroidectomized rat, hepatic nuclei with diameters of only 6.0 μ are found after a considerable post-operative interval, whereas in normal controls or in thyroidectomized animals treated with growth hormone, thyroxin, or both, nuclei with diameters of 6.5 and 8.0 μ are seen. The probability that growth hormone secretion is depressed after thyroidectomy could also explain our own unpublished findings that in animals which had been thyroidectomized at 24 days of age, the distribution of hepatic parenchymal cell types found at 114 days was that characteristic of 40- to 45-day-old animals. The change, in the normal animal, in the distribution of cell types with age has been previously reported (Alfert and Geschwind, 1958).

In order to eliminate the possibility that thyroxin was acting to enhance polyploidization by repairing pituitary function, experiments were conducted in hypophysectomized rats. Such animals have a further advantage of being far more sensitive to thyroxin than is the dwarf mouse, permitting a much smaller dose of thyroxin to be employed (2 micrograms daily to a rat weighing 75 grams versus 50 micrograms twice weekly to a mouse weighing 6 grams). Thus, any effects due to conjugation and metabolism by the liver, or to toxicity, are minimized.

The results of these experiments (Table II) reveal that the effect of thyroxin on the hypophysectomized rat is comparable, at the dose levels chosen, to that

obtained with growth hormone. In both cases a 25% decrease in the number of mononucleate diploid cells has occurred, along with a 5-fold increase in the number of tetraploid cells. As in the case of the dwarf mouse, the greatest effect, however, was found in those animals receiving both hormones, for in the livers of such animals the total number of diploid cells was only one-third that found in the control animals, while the numbers of both tetraploid and octaploid cells increased markedly. The synergistic effect of the two hormones administered together is also reflected in the body weight increments of the treated animals.

These experiments therefore indicate that thyroxin (and presumably thyrotropic hormone) as well as growth hormone contributes to the hormonal environment which affects the development of hepatic polyploidy. It should also be noted that thyroxin alone can be at least as efficient as growth hormone in promoting the progression of hepatic polyploidy; thus, as we also have demonstrated by the results of a previous experiment (Geschwind, Alfert and Schooley, 1958), the suggestion of Leuchtenberger *et al.* (1954), concerning the supposed essential role of growth hormone in somatic polyploidy, cannot be maintained.

Summary

1. The effect of thyroxin and of growth hormone on liver polyploidy has been investigated in the dwarf mouse and in the hypophysectomized rat.

2. Either hormone, acting alone, stimulated polyploidization in these experimental animals; the combination of both hormones synergized to produce an hepatic cell picture dominated by higher polyploid classes.

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SURFACE ANTIGEN DYNAMICS IN THE SLIME MOLD, DICTYOSTELIUM DISCOIDEUM [1]

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The mechanisms by which cells in a morphogenetic system effect coordinated movements and specific associations constitute basic developmental problems that have been the subject of many investigations. That such mechanisms possibly involve forces similar to those existing between antigens and antibodies was recognized and elaborated by Tyler (1947, 1957), Weiss (1947), and Spiegel (1954a, 1954b). A recent comprehensive review of the problems involved in such morphogenetic processes as cell adhesion and cell migration has been presented by De Haan (1958) and need not be recapitulated here.

Morphogenetic expression in the slime mold, *Dictyostelium discoideum*, requires oriented movements of cells, a process which involves changes in shapes of the cells. The stimulus for the changes in shape may be an external one (via acrasin) in which large numbers of cells are affected from a common direction simultaneously (Bonner, 1947, 1950). Following the aggregation process the cells are organized into a pseudoplasmodium in which the cell surfaces are in intimate association with each other. Throughout morphogenetic movements the cells maintain contact with each other. Acquisition of the ability to adhere to one another implies a change in the nature of the surface of the cells. Consistent with this is the evidence obtained by Gregg (1956) that new antigens appear at the onset of aggregation.

It is the purpose of this study to investigate changes in the surface antigens of the cells from the beginning aggregation stage to the formation of the mature spores. Such a study might be expected to reveal not only the pattern of antigens during morphogenesis but something of the possible role of surface antigens in maintenance of the integrity of the pseudoplasmodium and in regulation of the course of cellular movement.

Methods and Materials

The slime molds were cultured according to the general methods employed by Bonner (1947) with the exception that the bacterial associate was *Aerobacter aerogenes* rather than *Escherichia coli*. The cultures were maintained at 22° C. during the growth phase. During the period of aggregation they were transferred to a 17° C. incubator, the lower temperature of which extended the migration and culmination phases.

[1] This investigation was supported in part by a research grant E-1452 from the National Institutes of Health, Public Health Service.

Preparation of injection-antigens

Migrating pseudoplasmodia were collected by cutting discs of nutrient agar bearing aggregates on the verge of migration. These discs were transferred to similar sized spaces in plates on non-nutrient agar (Gregg and Bronsweig, 1956). After the migrating pseudoplasmodia had accumulated on the non-nutrient surface they were collected individually with a hair loop and transferred into 1.0% NaCl.

The mature spores were collected by sweeping the tops of the mature sorocarps with a 3-mm. glass rod. The spores adhered to the glass rod and were transferred into 1.0% NaCl.

An antigen was also prepared by washing mature spores in 0.5% NaCl for 10–15 minutes. The spores were then centrifuged and the supernatant carefully removed to serve as an antigen.

These collecting procedures enabled migrating pseudoplasmodia, mature spore, and mature spore surface antigens to be obtained essentially free of *Aerobacter*. The antigens were stored in 1.0-ml. aliquots in a deep freezer at minus 15° C.

Each of the three types of antigens contained 0.2 mg. N/ml. Two rabbits each were injected with the three types of antigens. The injections were given every other day until a total of nine injections per rabbit had been given (0.5 ml. antigen/injection).

Preparation of antisera and normal (pre-injection) sera

The rabbits were bled by heart puncture five days following the final injection. The blood was allowed to stand for approximately two hours at room temperature before the clot was ringed, then placed in the refrigerator overnight before centrifugation. Complement was destroyed by heating the antisera at 56° C. for 30 minutes. The antisera were divided into 1.0-ml. aliquots and placed in the deep freezer at minus 15° C.

The normal (pre-injection) sera were obtained and prepared in the same manner as described for the antisera.

Preparation of absorbed antisera

The amoebae or spores were harvested from the culture plates by the methods described under the sections concerning the preparation of injection-antigens and test-antigens. However, in the absorption procedure all of the supernatant was removed from the packed cells. In a typical absorption, packed cells were mixed with the particular antiserum in the ratio of one volume of cells to one volume of antiserum. The preparation was allowed to absorb at room temperature for two hours followed by 22–24 hours at 10° C. The absorbed serum was then centrifuged in an electric micro-centrifuge (Microchemical Specialties Co.) at 9500 rpm for 15 minutes. The serum was then carefully removed from the packed cells and stored at minus 15° C. until desired.

Preparation of test-antigens for agglutination

These antigens were prepared by alternately washing and centrifuging (125 *g*) aggregating amoebae or spores twice in 0.5% NaCl. The resulting sedimented

cells were diluted with about one volume of 0.5% NaCl (approximately 2.5×10^8 cells/ml.) for use in agglutination tests. The cells were placed in agglutination chambers in 10.0-μl. volumes (Gregg and Trygstad, 1958). The antiserum or normal (pre-injection) serum was mixed with the cells in 10.0-μl. volumes with

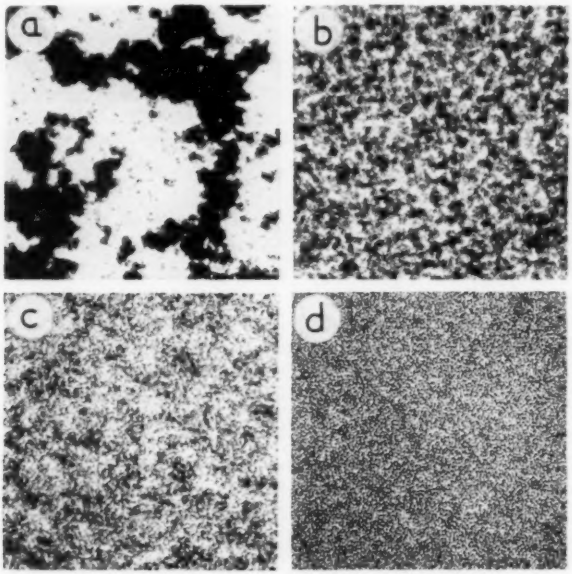

FIGURE 1a. *D. discoideum* amoebae + spore antiserum (magnification × 36). 1b. *D. discoideum* amoebae + spore normal serum (magnification × 36). 1c. *D. discoideum* spores + spore antiserum (magnification × 100). 1d. *D. discoideum* spores + spore normal serum (magnification × 100).

a small glass rod. The presence or absence of antibodies in the serum was detected by agglutination of the amoebae or spores to various degrees (Fig. 1a, b, c, d).

All antisera and normal (pre-injection) sera were tested on amoebae or spores independently. However, the results obtained from antisera produced in response

TABLE I

Antiserum titer obtained by agglutination of cells

Type of antiserum	Type of cell agglutinated	Highest dilution of antiserum
MA	Amoebae	1:80
SA	Amoebae	1:80
SSA	Amoebae	1:20

to the same type antigen or normal (pre-injection) sera have been pooled. The titers of the various sera were obtained by determining the least concentration of antiserum diluted with 1.0% NaCl, which would produce detectable agglutination of amoebae or spores (Table I).

Determination of the amounts of antigenic material extracted from the surfaces of the cells

For reasons which will become apparent it was necessary to determine quantitatively the amounts of antigenic material which may be extracted from the surfaces of the cells composing migrating pseudoplasmodia and the mature spore masses. These determinations were made in two ways as follows:

A. 1. Ten to fifty migrating pseudoplasmodia (non-nutrient agar migrants) or mature spore masses placed in 20 μl. of 0.5% NaCl.

2. Cell surface materials extracted by gentle stirring for 10–15 minutes.

3. Cells centrifuged 5 minutes at 4600 rpm in the micro-centrifuge.

4. Washed cells and surface wash analyzed separately for total N (Bruel *et al.*, 1946; Gregg, 1950) (Table IV).

B. 1. One hundred to five hundred migrating pseudoplasmodia or mature spore masses were placed in 100–500 μl. 1.0% NaCl.

2. Cell surface materials extracted by gentle stirring for 10–15 minutes.

3. Cells centrifuged 15 minutes at 9500 rpm in the micro-centrifuge.

4. The surface wash was removed and saved for analysis.

5. The washed cells were analyzed for total N.

6. The volume of each surface wash from each type of cell was adjusted such that $\frac{\mu\text{l. surface wash}}{\mu\text{g. N of washed cells}} = \text{unity}$. This dilution procedure resulted in the production of surface wash in volumes proportional to the number of cells (total N) from which it was extracted.

7. The surface washes were then subjected to conventional procedures to determine optimum proportions with various antisera in precipitin tests.

8. Fifty μl. of each surface wash and the optimum amount of each antiserum, determined in step 7, were mixed in 3 mm. × 40 mm. test tubes and allowed to incubate for 1–2 hours at room temperature. After incubation in a refrigerator overnight the precipitates were centrifuged at 9500 rpm for 15 minutes in the micro-centrifuge. The precipitates obtained were washed once with 1.0% NaCl and analyzed for total N (Table V).

Results

Migrating pseudoplasmodia-antiserum (MA)

The MA effected maximal agglutination of both amoebae and spores (Table II). It should be noted here, however, that spores do not exhibit the massive type of agglutination obtained with amoebae. The maximal agglutination in spores amounts to an easily detectable regular pattern of small clumps of spores, but never the large masses of drastically clumped cells as observed when agglutinating amoebae.

Migrating pseudoplasmodia-antiserum absorbed with amoebae (MAa)

The MAa did not agglutinate amoebae or spores (Table III).

Migrating pseudoplasmodia-antiserum absorbed with spores (MAs)

The MAs did not agglutinate amoebae or spores (Table III).

Migrating pseudoplasmodia normal (pre-injection) serum (MN)

The MN used as controls in all agglutination tests along with MA, MAa, and MAs, did not effect agglutination of either amoebae or spores (Tables II, III).

Mature spore-antiserum (SA)

The SA agglutinated both amoebae and spores to the degree designated as maximum for each type of cell (Table II) (Fig. 1a, c).

TABLE II

Degree of agglutination effected by various antisera and normal (pre-injection) sera on amoebae and spores

+ = maximum agglutination; ± = minimal agglutination; and − = no agglutination. All sera were diluted to one-half of full strength as a result of the addition of amoebae. The numbers opposite the degree of agglutination values refer to the number of times a particular pattern was observed.

	Type of sera	Amoebae Degree of agglutination			Spores Degree of agglutination*		
		+	±	−	+	±	−
Antisera	Migrating	49				26	
	Spore	40				24	
	Spore surface	21				13	
Normal sera	Migrating			48			24
	Spore			39			22
	Spore surface			19			15

* In this table and Table III, ± represents the maximal degree to which spores may be agglutinated.

Mature spore-antiserum absorbed with amoebae (SAa)

The SAa failed to agglutinate amoebae, but effected maximal agglutination of spores (Table III).

Mature spore-antiserum absorbed with spores (SAs)

The SAs failed to agglutinate either amoebae or spores (Table III).

Mature spore normal (pre-injection) serum (SN)

The SN used as controls in all agglutination tests along with SA, SAa, and SAs did not effect agglutination of either amoebae or spores (Tables II, III) (Fig. 1b, d).

Mature spore surface antiserum (SSA)

The SSA effected maximal agglutination of both amoebae and spores (Table II).

TABLE III

Degree of agglutination effected by various absorbed antisera and normal (pre-injection) sera on amoebae and spores

+ = maximum agglutination; ± = minimal agglutination; and − = no agglutination. All sera were diluted to one-half of full strength as a result of the addition of amoebae. The numbers opposite the degree of agglutination values refer to the number of times a particular pattern was observed.

	Type of sera	Amoebae Degree of agglutination			Spores Degree of agglutination*		
		+	±	−	+	±	−
Antisera abs. with amoebae	Migrating			26			10
	Spore			24		11	
	Spore surface			3		3	
Antisera abs. with spores	Migrating			9			4
	Spore			14			4
	Spore surface			7			3
Normal sera	Migrating			34			12
	Spore			38			12
	Spore surface			10			4

* See footnote to Table II.

TABLE IV

Comparison of total N in surface antigens of migrating pseudoplasmodia amoebae and mature sorocarp spores

Cell type	No. of exps.	μg. whole cell N	μg. surface antigen N	$\frac{\mu g. \text{ surface antigen N}}{\mu g. \text{ whole cell N}}$	Ratio of antigen N $\frac{\text{Spore}}{\text{Amoebae}}$
Spores	6	133.3	31.0	0.23	1.5
Amoebae	4	53.9	8.0	0.15	

TABLE V

Comparison of total N precipitated from surface antigen preparations of migrating pseudoplasmodia amoebae and mature sorocarp spores by antibodies

	No. of exp.	Surface antigens obtained from		Ratio of precipitate N $\frac{\text{Spore}}{\text{Amoebae}}$
		Amoebae	Spores	
μg. N precipitated by spore surface antiserum minus controls (normal serum)	3	6.8	30.0	4.4
μg. N precipitated by migrating pseudoplasmodia antiserum minus controls (normal serum)	3	3.0	8.0	2.7

Mature spore surface antiserum absorbed with amoebae (SSAa)

The SSAa failed to agglutinate amoebae but effected maximum agglutination of spores (Table III).

Mature spore surface antiserum absorbed with spores (SSAs)

The SSAs failed to agglutinate either amoebae or spores (Table III).

Mature spore surface normal (pre-injection) serum (SSN)

The SSN did not effect agglutination of either amoebae or spores (Tables II, III).

The amounts of surface material removed from cells composing migrating pseudoplasmodia and mature spores

It was found that in terms of total N of the surface washes, 1.5 times more material was washed from the cells composing the spore masses than from the amoebae composing migrating pseudoplasmodia (Table IV). It was assumed that the N content of the surface washes would serve as an indicator of the amount of antigenic or surface material which had been removed from the cells.

When surface washes of the two types of cells were precipitated with antisera the precipitates obtained from spore surface wash proved to be 2.7–4.4 times greater than those from amoebae surface wash (Table V). This indicates that more antigenic material was removed from the cells composing spore masses than from those composing migrating pseudoplasmodia.

Discussion

Antisera produced in rabbits to antigens composed of cells and washings of cells from various stages of the slime mold, *D. discoideum*, effect agglutination of amoebae and spores. Analysis of the pattern of agglutination effected by the various types of sera on amoebae and spores demonstrated that qualitatively identical surface antigens are present during the transition from the beginning aggregate to the mature spore. However, absorption of various antisera by amoebae and spores has disclosed the presence of a surface antigen(s) in the spores which is (are) not common to early aggregates or migrating pseudoplasmodia. It is possible that the new antigen is composed of the polysaccharide with which spores become coated during the culmination process (Raper and Fennell, 1952).

An examination of Figure 1 reveals that the maximal degree to which amoebae may be agglutinated differs from that degree to which spore cells may be agglutinated. It may be seen that spores agglutinate poorly even with mature spore antiserum as compared with the agglutination of amoebae by the same antiserum.

Shaffer (1958) has demonstrated that *D. discoideum* amoebae become strongly adhesive at the onset of aggregation. Furthermore, it has been observed during the course of the present work that the amoebae composing a migrating pseudoplasmodium do not spread readily upon a glass rod. The failure to spread has been interpreted as being due to the adherence of the cells to each other. However, the cells composing a spore mass tend to spread readily when tested in the same fashion, suggesting that the adhesive properties of the spores are relatively poor.

In seeking an explanation for the particular serological and adhesive properties exhibited by the amoebae and spore cells it was found that surface materials obtained by washing cells in 0.5% NaCl were antigenic (ring and precipitin tests) with MA and SA. Such preparations were subjected to two types of analyses to compare quantitatively the amounts of material which had been extracted from the amoebae and spore cell surfaces. These determinations revealed that greater amounts of antigenic material were found in spore wash as compared to amoebae wash (Tables IV, V). To account for the difference in the amount of antigenic material recovered from surface washings of amoebae and spores it is suggested that an increase occurs in the amount of loosely bound surface antigen on the spores. Another possibility would include an elimination of the antigens from the pre-stalk and pre-spore surfaces as a consequence of their role in morphogenetic movements during the culmination process. Antigens ejected from the cell surfaces would be contained in the spore extracellular spaces. A mechanism by which surface antigens are discharged and renewed at the surfaces of the cells would result in the reversibility of cell adhesiveness. Such reversibility is conceivably necessary if cells in contact with each other are to retain their motility.

Upon injecting spore washings into rabbits, antiserum was produced which was capable of agglutinating both amoebae and spores (Table II). Such antiserum (SSA) when subjected to absorption procedures with amoebae or spores reacted with test cells in the same way as the antiserum (SA) produced by injecting whole spores into rabbits (Tables II, III).

The fact that antiserum obtained from whole spore injection cannot be distinguished from spore surface wash antiserum suggests that the same antigens were involved in the production of each antiserum. The ease with which spore surface wash antigen may be removed implies that it is either loosely bound to the cell surfaces or was discharged from the cells during morphogenesis. Therefore, it seems plausible to attribute the poor agglutination of the spores to the inability of the antigens, with which the antibodies combine, to remain attached to the cell surfaces. Consequently, a reduction in the number of antigenic sites involved in effecting agglutination of cells in the presence of antisera would occur. Few antigenic sites on the spore surfaces would probably suffice for the production of antisera in rabbits to those particular antigens, which accounts for the agglutinating ability of the spore antiserum (SA) on amoebae or spores. Another possibility exists that the poor agglutination of the spores might be attributed to the relative inaccessibility of antigenic sites on spore surfaces. Such an interpretation, for the failure of ox red cells to agglutinate in the presence of certain antisera, was advanced by Coombs *et al.* (1951). The existence of this type of cell surface configuration of the spores could conceivably be tested by Coombs *et al.*'s method.

The poor agglutinating capacity of spores is paralleled by weak adhesiveness between the spores. It is interesting to point out that relatively large amounts of antigen appear in washings of spore cell surfaces. These facts suggest that the particular agglutinating and adhesive properties of the spores are related to the appearance of antigen in spore washings.

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versity of Florida College of Medicine, for his technical assistance during the course of this investigation.

Summary

1. Methods are described whereby antigenic material of the slime mold, *D. discoideum*, effective in antibody production in rabbits, is prepared.

2. Antisera produced in response to migrating pseudoplasmodia, mature spores and mature spore surface antigens effected maximal agglutination of amoebae. Such antisera effected only minimal agglutination of spores.

3. Antisera of the above types absorbed by amoebae and spores in all combinations revealed the presence of a surface antigen(s) on the spores which is (are) not common to amoebae from early aggregates or migrating pseudoplasmodia.

4. It has been suggested that a relationship exists between the adhesive properties of the amoebae and spores and their agglutinating properties in the presence of antisera.

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THE EFFECT OF POTASSIUM DEFICIENCY ON THE FREE AMINO ACID PATTERN OF THE MUSCLE TISSUE OF PROTEIN-MAINTAINED FUNDULUS HETEROCLITUS [1, 2]

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In this investigation the effect of a low potassium environment on the free amino acid pattern of the skeletal muscle tissue of the killifish, *Fundulus heteroclitus*, has been studied in an attempt to determine if the changes observed in the potassium-deficient rat (Eckel, Pope and Norris, 1954; Iacobellis, Muntwyler and Dodgen, 1956) could also be found in other members of the vertebrate subphylum. Apparently the rat, but not the dog (Iacobellis, Griffen and Muntwyler, 1957), is capable of replacing part of an intracellular loss of potassium ions with organic cations in the form of basic amino acids, provided a sufficient amount of protein is included in the diet. Amino acids which could serve to restore an intracellular cation-anion balance include lysine, arginine, and histidine. All three carry a net positive charge at body pH. This study, therefore, was made to determine if an intracellular organic cation replacement occurs in potassium-deficient *Fundulus* and, if so, which basic amino acids are responsible for the replacement of the lost potassium.

Methods

Killifish (*Fundulus heteroclitus*) were trapped in the brackish water of the Oyster River near Durham early in August of 1958 and transported to the laboratory where they were placed in a large aquarium containing artificially prepared 50% sea water (salinity = 18 parts per thousand). The formula followed was taken from the Marine Biological Laboratory at Woods Hole manual, "Formulae and Methods, IV," 1954. After a three-day period of adjustment, one-half of the animals was left in 50% sea water to serve as controls, whereas the other half was placed in an adjacent aquarium also containing 50% artificial sea water but lacking potassium ions. Control and potassium-deficient animals were maintained on a diet of powdered, crude casein.

At the end of a two-week period animals were removed from each aquarium and their tissues prepared for paper chromatography. In order to obtain 10 grams of skeletal muscle tissue in each case it was necessary to sacrifice 7 control *Fundulus* and 8 potassium-deficient *Fundulus*. The fish were skinned and filleted. Ten grams of muscle were macerated in a Waring Blendor, using about 100 ml. of 95% ethanol. After 5 minutes of blending at a moderate speed the liquid was

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transferred to 50-ml. centrifuge tubes and centrifuged for 5 minutes at 3000 r.p.m. The centrifugate was removed (the precipitate consists chiefly of protein) and reduced *in vacuo* to a volume corresponding to 1 ml. per gm. of tissue extracted. Slight heat was applied to speed reduction. While awaiting use, the extract samples were kept in the refrigerator.

Two-dimensional ascending paper chromatography was employed to determine in a semi-quantitative manner the free amino acids present in the muscle tissue of the control and potassium-deficient *Fundulus*. Aliquots of 60 μl. were spotted on Whatman No. 1 filter paper sheets (9″ by 12″) and developed for 16 hours with a phenol-water (4:1) solvent. The chromatograms were dried at room temperature and developed for 16 hours in the second dimension with a lutidine-water (2:1) solvent. After drying, the amino acids were located by spraying the chromatograms with a 0.1% solution of ninhydrin in 95% ethanol. The Sakaguchi test for arginine and the Pauli test for histidine were also employed (Block, Durrum and Zweig, 1955). The location of lysine was confirmed on the ninhydrin-developed chromatograms by exposing them to hydrochloric acid fumes for one minute. The spot representing lysine turns a bright yellow (Szantai, 1957) which is stable for several days. Valine, leucine and threonine also turn yellow, but the color soon fades. This, taken in conjunction with the relative positions of the above-named acids on the two-dimensional chromatogram, makes any confusion of valine, leucine or threonine for lysine unlikely.

Total amino acid concentration was determined quantitatively as follows. Ten μl. of the tissue fluid extracts were spotted on Whatman No. 1 filter paper. Also spotted on the filter paper were 10 μl. quantities of a standard solution containing 6 μgm. of amino acid per μl. Since three amino acids, glycine, alanine and taurine, were found to be responsible for the bulk of the color observed on the ninhydrin-sprayed chromatograms, the standard was prepared by dissolving 20 mg. each of these amino acids in 10 ml. of distilled water. It was felt desirable to use this type of standard to allow for differences in the absorption characteristics of the ninhydrin complexes. After application the spotted amino acid solutions were allowed to dry at room temperature, then were sprayed with a 0.5% solution of ninhydrin in 75% ethanol plus 0.5% 1 N sodium hydroxide. Kay, Harris and Entenman (1956) report that the presence of sodium hydroxide is essential for the development of color in the case of taurine, but otherwise has no effect. Curiously, the author found that sodium hydroxide had no such specific enhancing effect in the case of taurine, although a general increased color density was observed. The ninhydrin-treated filter paper was dried at room temperature and then heated at 60° C. for 25 minutes. The colored areas were cut out and eluted with 5 ml. of a 75% ethanol solution containing 10 mg. of cupric sulfate ($CuSO_4 \cdot 5H_2O$) in 200 ml. of solution (Giri, Radhakrishnan and Vaidyanathan, 1952). Blanks were prepared by cutting out portions of filter paper equal in area to those supporting the colored complexes. Optical density of the colored solutions was read at 540 mμ on a Bausch-Lomb "Spectronic 20."

Two-dimensional paper chromatography was used to determine the amount of lysine present in the muscle tissue of potassium-deficient and control *Fundulus*. Sixty-μl. aliquots were used in the case of the potassium-deficient fish whereas 400 μl. were required in the case of the control animals, due to the very low con-

centration of lysine in their tissues. Three to 5 μl. of an aqueous solution containing 6 mg. of lysine per ml. of solution, serving as standards, were spotted and chromatographed. Chromatograms were developed using phenol-water (4:1) and lutidine-water (2:1) as solvents. After drying at room temperature the chromatograms were sprayed with ninhydrin as before. Apparently the spraying procedure is the most crucial point in the quantitative determination. Care should be taken to spray the chromatograms lightly and evenly on one side, allow them to dry, then repeat the operation on the other side. The chromatograms were heated, lysine spots cut out and eluted and optical density read as in the case of the determination of total amino acid concentration.

Results

Fifteen amino acids were found to be present in the extracts of the control and potassium-deficient animals. The same amino acids were present in both cases. Furthermore, it was found that (1) the total amino acid concentration

Table I

The total concentration of amino acids and the concentration of lysine in the skeletal muscle of control and potassium-deficient Fundulus heteroclitus

	Total concentration (in mg./gm. of tissue)		Lysine concentration (in mg./gm. of tissue)	
	Potassium-deficient *Fundulus*	Control *Fundulus*	Potassium-deficient *Fundulus*	Control *Fundulus*
Range	4.9–6.2	2.7–3.3	0.32–0.42	0.030–0.035
Mean	5.4*	3.0*	0.38†	0.033†
No. of determinations	13	17	4	3

* Difference is significant at 0.1% level.
† Difference is significant at 1.0% level.

in the tissues of the potassium-deficient *Fundulus* appeared to be somewhat greater than in the controls (Table I), and (2) the concentration of lysine in the potassium-deficient fish was much greater than in the controls, whereas the levels of histidine and arginine did not seem to be disproportionately large in the former. It was felt that the apparently greater total amino acid concentration observed in the potassium-deficient animals could not account for the extreme differences seen in the concentration of lysine.

Table I shows the values obtained from 17 determinations of the total amino acid concentration in the tissues of the controls and 13 determinations of the potassium-deficient fish. A certain amount of variation occurs in both cases. However, the extent of variation is relatively small when the difficulty of the method is considered. As was suggested by the semi-quantitative determination, a significantly greater concentration of free amino acids occurs in the muscle tissues of the potassium-deficient *Fundulus*. Although in the controls the concentration

averages 3.0 mg. per gm. of tissue extracted, it reaches a level of 5.4 mg. per gm. in the potassium-deficient forms, *i.e.*, a concentration 1.8 times as great.

Table I indicates the values obtained for lysine levels in control and potassium-deficient *Fundulus*. Although only three determinations were made on the control animals and four on the potassium-deficient animals, the degree of variation was small in both instances and the differences observed in the lysine concentrations are regarded as significant. Indeed, the concentration of lysine in the skeletal muscle of the potassium-deficient forms appears to be more than 10 times as great as that of the control animals, whereas the total amino acid concentration is only 1.8 times as great.

Discussion

If animals are made deficient in potassium an influx of available extracellular cations would be expected in order to restore the cation-anion balance. In certain animals the lost potassium can be partly replaced by a gain in sodium. According to Cooke and his co-workers (1952) the remainder is replaced by an intracellular migration of protons, the result being the development of a plasma alkalosis. However, several investigators (Muntwyler, Griffen and Arends, 1953; Iacobellis, Muntwyler and Dodgen, 1956; Holliday and Segar, 1957) observed no plasma alkalosis in the potassium-deficient rat. Since Christensen, Riggs and Palatine (1952) showed that basic amino acids could act as intracellular cations, Eckel and his co-workers (1954) investigated the possibility of this being the case in potassium-deficient rats demonstrating no plasma alkalosis. Their results revealed that 8 to 40% of the intracellular metallic cation deficiency is replaced by lysine.

The results of this investigation show that, as in the case of the rat, the cation-anion imbalance appearing in the potassium-deficient *Fundulus* is partially overcome by an increase in the intracellular concentration of lysine. In Table I it can be seen that the concentration of lysine in the potassium-deficient *Fundulus* is approximately 10 times greater than in the controls. In terms of per cent of total amino acid concentration, the value for lysine is 1.1% in the case of the control animals and 7.0% in the potassium-deficient animals, an increase greater than 500%.

The present results indicate that histidine and arginine contribute little to the increase in intracellular organic cation concentration in *Fundulus*. Furthermore no decrease was observed in aspartic and glutamic acids as Iacobellis, Muntwyler and Dodgen (1956) found with Wistar rats.

The total free amino acid concentration in the skeletal muscle tissue of potassium-deficient *Fundulus* is almost twice that of the controls (Table I). The observed increase may be the result of a decrease in the rate of nitrogen anabolism and perhaps the establishment of a negative nitrogen balance. Evidence that such is the case here is supplied by the work of Muntwyler, Griffen and Arends (1953) and Eckel, Norris and Pope (1958). Both groups reported decreased growth and a failure to maintain a proper nitrogen balance in the potassium-deficient rat. Conceivably in *Fundulus* as well as the rat an increased amount of free amino acids would be due to a failure of body protein synthesis and in certain cases (lysine) could serve to ameliorate a state of cation-anion imbalance by functioning as intracellular cations.

The fact that an identical device for combatting a loss of cellular potassium can be seen in animals as widely separated as mammals and fish suggests the

interesting possibility of the universality of such a mechanism. It may be that a similar or perhaps identical mechanism accompanying a potassium deficiency can be found throughout the entire vertebrate group, indeed, perhaps in several phyla of the animal kingdom.

Summary

1. The effect of a potassium deficiency on the free amino acids of the muscle tissues of *Fundulus heteroclitus* was investigated employing the method of quantitative paper chromatography.

2. An over-all increase in the total amino acid concentration was observed in the potassium-deficient fish amounting to 1.8 times that seen in the controls. A great increase in the concentration of lysine was also observed in the potassium-deficient forms. In this case the concentration was about 10 times that of the controls.

3. It is therefore suggested that the mode of combating a potassium deficiency in the rat also exists in a fish. The interesting possibility that similar or perhaps identical mechanisms for lessening the effects of a potassium deficiency may be found throughout other representative vertebrate groups, as well as the invertebrates, offers a fruitful field for further comparative investigations.

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STRUCTURAL AND FUNCTIONAL CHANGES IN A GENERATION IN TETRAHYMENA[1]

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With the development of techniques for synchronizing the division of members of a population of microorganisms, it has become possible to analyze many changes that occur in a cell during a generation (the period from the end of one division to the end of the next). The ciliate protozoan *Tetrahymena pyriformis* has been used in this way by Zeuthen and his coworkers in studies directed at biochemical changes; nitrogen content (Hamburger and Zeuthen, 1958), nucleoside triphosphate concentration (Plesner, 1958a), rate of incorporation of P^{32} (Hamburger and Zeuthen, 1958), and sensitivity to anaerobiosis (Rasmussen, 1958), dinitrophenol (Hamburger and Zeuthen, 1957), and inhibitors of oxidative metabolism (Hamburger, 1958). Synchronously-dividing populations are also useful for tracing the time course of structural changes. *Tetrahymena* is uniquely suited to such a study since it passes through a period of morphogenesis in each generation that results in duplication of the structures necessary for the formation of a new individual before division (body cilia, mouth, contractile vacuoles, cytopyge) (Chatton *et al.*, 1931; Furgason, 1940), and it undergoes nuclear changes involving a mitotic division of the micronucleus and an amitotic division of the macronucleus (Maupas, 1883, 1888).

The following is a description of the time course of the structural changes (those visible with the light microscope) in a generation, in a synchronized population of *Tetrahymena pyriformis*, mating type I, variety 1, and a comparison of it with the time course of known chemical changes in a generation, observed with other strains of *Tetrahymena*.

Materials and Methods

Division was synchronized by the application of 5 alternate ½-hour exposures to temperatures of 42.6 ± 0.2° C. and 35 ± 0.2° C. Other details of procedure were as described by Holz, Scherbaum and Williams (1957). Following the final high temperature period, samples of ciliates were withdrawn at 5-minute intervals for 180 minutes. To determine when the first and second synchronous divisions occurred, the division index (cells in division ÷ cells counted) was measured for each sample. The first synchronous division (70–80% synchrony) occurred 50–60 minutes after the last high temperature period, and the second division (45–55% synchrony) 65–75 minutes after the first division. Changes in the nuclear apparatus (Fig. 1), infraciliature (Fig. 8), and general appearance of the ciliates during the interval between divisions were followed by Feulgen-staining (Dippell

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and Chao's modification of the DeLamater stain; Sonneborn, 1950), silver impregnation (Chatton-Lwoff technique; Corliss, 1953), and the observation of living individuals with a phase contrast microscope. The temporal positions of the nuclear and morphogenetic events in a generation were determined by comparisons of the times of greatest frequency of appearance of these events (in the samples taken between the first and second synchronous divisions) with the times when the first and second synchronous divisions occurred.

To establish a basis for comparison of the times of structural changes in mating type I, variety 1, with the times of changes in physiological and biochemical parameters reported for other strains, all generation times of the various strains used were compressed to 100 arbitrary units and the time courses of all changes adjusted to fit this scale. For synchronously-dividing cells, the generation time used in these calculations was that of the synchronous generation during which observation of a particular structural or functional change was made. With one exception this was the second synchronous generation (the period between the first and second synchronous divisions). Nucleoside triphosphate content was determined at various times during the first synchronous generation (the period from the end of the last high temperature exposure to the first synchronous division) (Plesner, 1958a). Data on DNA synthesis (McDonald, 1958) and sensitivity to extremes of temperature (Thormar, 1956) were obtained with single normal cells, nitrogen content (Hamburger and Zeuthen, 1958), sensitivity to dinitrophenol (Hamburger and Zeuthen, 1957), sensitivity to inhibitors of oxidative metabolism (Hamburger, 1958), nucleoside triphosphate content (Plesner, 1958a), and rate of incorporation of P^{32} (Hamburger and Zeuthen, 1958) were measured with groups of synchronized cells, and rate of respiration (Zeuthen, 1953; Zeuthen and Scherbaum, 1954) and sensitivity to anaerobiosis (Rasmussen, 1958) with both types. Such a comparison is based on several assumptions: first, that there are no gross differences in the time scales of structural and functional events in a generation in different strains of *Tetrahymena pyriformis* with different generation times and nuclear constitutions (micronucleate and amicronucleate), *i.e.*, that each event occurs at the same relative time in every strain; second, that the time characteristics of structural and functional changes occurring between synchronous divisions can be compared with those occurring between divisions of normal cells (cells that have not been heat-treated).

The first assumption is made on the basis that the structural and chemical changes that have been studied are of such a fundamental nature, necessary to the orderly progression of growth and preparation for division, that they probably follow the same relative time courses in all strains. This assumption was tested experimentally, so far as structural changes were concerned, by comparing the time courses of morphogenetic and macronuclear changes in strain GL and in mating type I, variety 1, with synchronized populations. Strain GL was synchronized by the method of Scherbaum and Zeuthen (1954). Comparison was also made of the time course of structural changes of mating type I, variety 1, with that reported by Browning, Varnedoe and Swinford (1952) for strain T-P. McDonald (1958) and Prescott and Bors (1958), using different strains, reached fundamentally the same conclusion concerning the period of DNA synthesis in a generation.

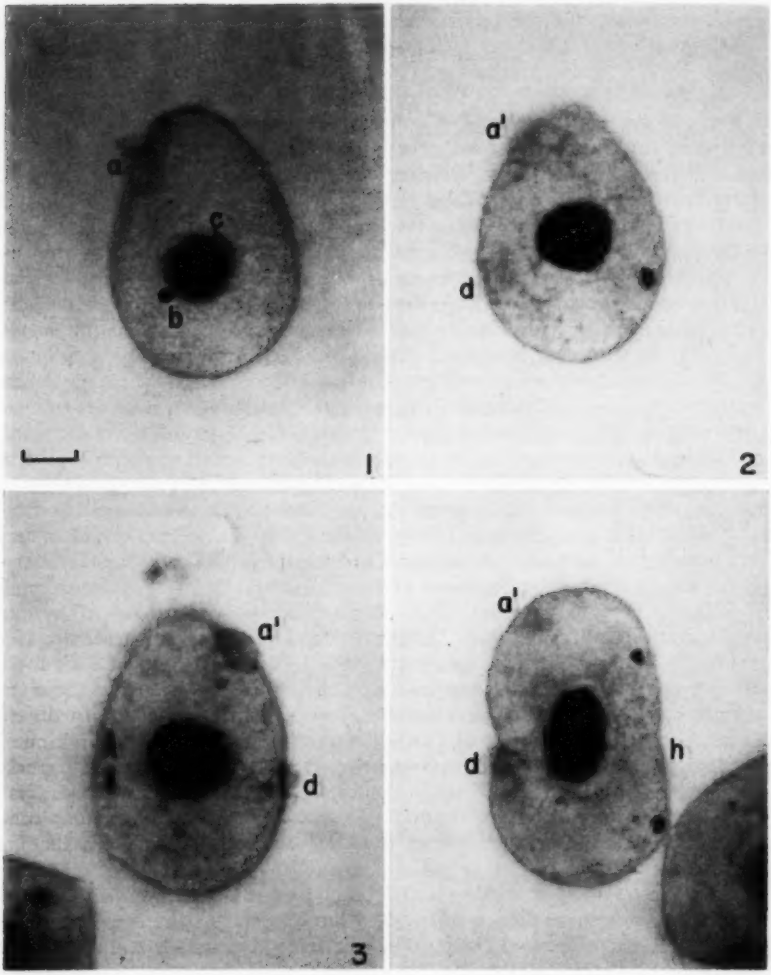

FIGURE 1. Resting nuclear configuration in *Tetrahymena pyriformis*, mating type I, variety 1. In this and the following illustrations of nuclear states, ciliates were not destained to a degree necessary to bring out fine nuclear detail. This was done to insure visualization of the cilia of the mouth, to assist in observing time relations of nuclear and morphogenic states. The marker indicates 10 μ for Figures 1–7.

FIGURE 2. Stage of macronuclear enlargement. The micronucleus is peripherally located. Cilia of the forming mouth of the opisthe are seen.

FIGURE 3. Micronuclear anaphase.

FIGURE 4. Micronuclear division complete. Macronuclear division and cytoplasmic cleavage beginning.

The second assumption is more open to question. Cells prepared for synchronous division by the temperature-cycling method have a shorter generation time (Zeuthen and Scherbaum, 1954), have a greater volume (Scherbaum, 1956), greater reduced weight (Scherbaum and Zeuthen, 1954), greater dry weight (Scherbaum, 1957b), and contain more nitrogen (Zeuthen and Hamburger, 1956), DNA and RNA (Scherbaum, 1957b), ATP and GTP (Plesner, 1956, 1958b) than do normal cells. During the synchronous divisions these characteristics return to normal. To liken the time courses of biochemical changes that have been studied in these cells to the condition in normal cells, we must assume that the differences noted above influence only the extent of the changes studied and not their time characteristics with respect to division. In this connection, it is notable that rate of respiration (Zeuthen, 1953; Zeuthen and Scherbaum, 1954) and sensitivity to anaerobiosis (Rasmussen, 1958) follow the same time courses between divisions in heat-treated and normal cells. The chemical composition of the heat-treated cells might cause the morphogenetic and nuclear changes to follow different time courses *with respect to one another*. To test this possibility, synchronized cells, and normal cells from cultures reproducing at an exponential rate, were compared for nuclear state in silver impregnations showing stages of stomatogenesis. In such preparations the micronucleus shows up well except during its final division stages.

Results

The resting micronucleus was spherical and lay next to the macronucleus, or within a cup-shaped depression in the macronucleus (Fig. 1). The resting state was assumed immediately after completion of cytoplasmic cleavage and lasted for about 75% of a generation. At the end of this period the micronucleus moved laterally toward the periphery of the organism and elongated (Fig. 2). Chromosomes appeared at this time. The most clearly recognizable stages of mitosis were those associated with separation of the chromosomes (Fig. 3). As the chromosomes reached the poles of the division figure the latter greatly elongated and the micronucleus was divided into halves which became separated respectively into the anterior and posterior parts of the dividing organism (Fig. 4). These daughter micronuclei then became spherical again. The division of the micronucleus went to completion as macronuclear division and cytoplasmic cleavage began.

Key to Figures 1–16

a Mouth of "resting" (interphase) ciliate.
a′ Mouth of proter (anterior daughter) during stomatogenesis.
b Micronucleus.
c Macronucleus.
d Mouth of opisthe (posterior daughter).
e Macronuclear fragment separated during macronuclear division.
f Stomatogenous field, site of formation of mouth of opisthe.
g Contractile vacuole pore of proter.
h Cleavage furrow.
i Stomatogenous meridian.
j Formation of membranelles.
k Formation of undulating membrane.

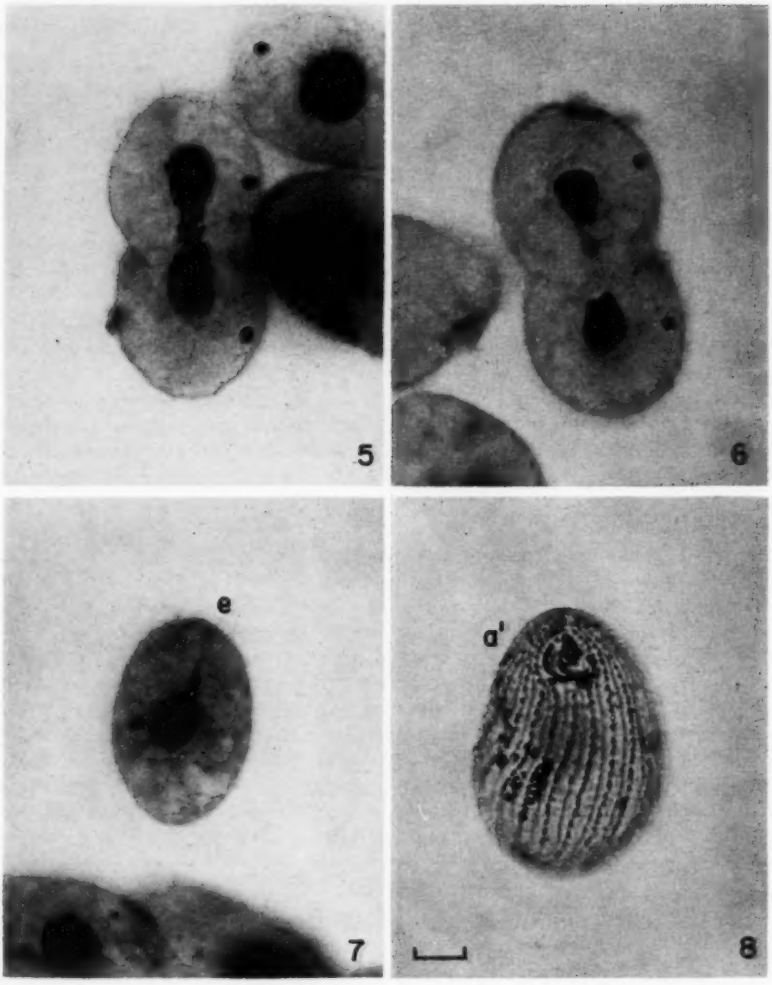

FIGURE 5. Macronuclear division and cytoplasmic cleavage continuing.
FIGURE 6. Macronuclear division complete. Cytoplasmic cleavage continuing.
FIGURE 7. Product of a recent division, showing re-establishment of resting nuclear configuration.
FIGURE 8. Infraciliature of *Tetrahymena pyriformis*, mating type I, variety 1. Note normal mouth of proter with typical tetrahymenal configuration, and stomatogenesis to produce mouth of opisthe. The marker indicates 10 μ for Figures 8–11.

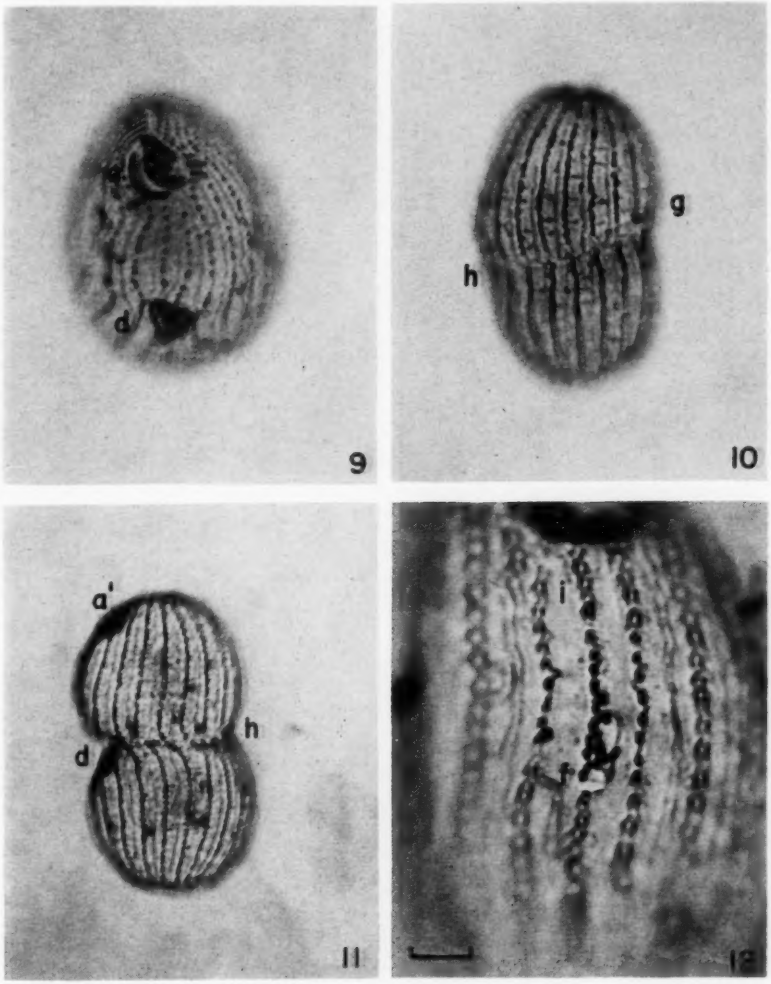

FIGURE 9. Completion of stomatogenesis in opisthe.

FIGURE 10. Appearance of cleavage furrow at beginning of division (dorsal surface of ciliate). Note contractile vacuole pore of opisthe just anterior to furrow on right side of animal.

FIGURE 11. Cleavage.

FIGURE 12. Very early stage of stomatogenesis. Kinetosomes appear to left (animal's left) of stomatogenous meridian. The marker indicates 5 μ for Figures 12–16.

FIGURE 13. Stomatogenesis; anarchic field.
FIGURE 14. Stomatogenesis. Very early stage of membranelle organization at anterior end of field.
FIGURE 15. Stomatogenesis. Three membranelles and undulating membrane formed.
FIGURE 16. Stomatogenesis; terminal stage.

The macronucleus was an approximately spherical, centrally-located structure (Fig. 1). Immediately after cleavage, it assumed a resting state characterized by a moderately dense and homogeneous granular appearance. At the time the micronucleus migrated to the periphery of the organism, the macronucleus in-

creased in size and granularity (Fig. 2). As the micronucleus divided, the macronucleus elongated (Fig. 4), and at the time the cleavage furrow separated the daughter ciliates, it divided into approximately equal halves (Figs. 5 and 6). Small portions of the macronucleus occasionally separated from the main macronuclear masses at this time and became isolated in the cytoplasm as spherical, Feulgen-positive bodies which were later resorbed (Furgason, 1940; Scherbaum *et al.*, 1958).

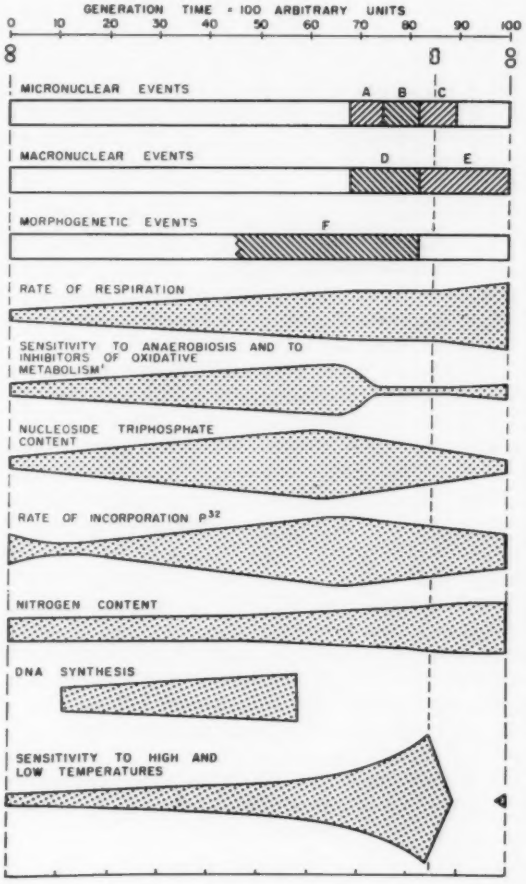

FIGURE 17. Graphic representation of approximate time relationships of structural and functional changes occurring in *Tetrahymena pyriformis* in a generation. (A—migration of micronucleus to periphery, B—mitosis, C—separation of products of mitosis into daughters, D—macronuclear swelling, E—macronuclear division, F—stomatogenesis, 1-nitrogen; Rasmussen, 1958, dinitrophenol; Hamburger and Zeuthen, 1957, fluoride, fluoracetate, azide, ethylurethane; Hamburger, 1958).

Morphogenesis in *Tetrahymena* involves formation of new body cilia, a new mouth (stomatogenesis) for the posterior daughter (opisthe), and new contractile vacuole pores and a cytopyge (cell anus) for the anterior daughter (proter). Body cilia are duplicated at a time not yet precisely determined. Stomatogenesis (mouth formation) was manifested first by the appearance of kinetosomes (basal bodies of cilia) to the left of the stomatogenous kinety (row of cilia, or primary ciliary meridian, passing posteriorly from the right side of the buccal overture) near the middle of the body (Fig. 8). The precise time when stomatogenesis began was not determined but early stages were seen before 45% of a generation was completed. Successive stages in the duplication of the kinetosomes of the cilia of the new mouth and their organization into membranelles are shown in Figures 12–16. The anlage of the mouth was in the late anarchic field stage (Fig. 14), or early stage of membranelle organization, at the time the micronucleus assumed a peripheral position. Stomatogenesis was complete, and the membranelles of the mouth appeared to be functional in living ciliates at the time of division of the micronucleus (Figs. 9, 15, 16).

The contractile vacuole pores of the proter were seen in silver impregnations (Fig. 10) as early as the time of lateral movement of the micronucleus, and the contractile vacuole was observed functioning in living organisms at this time.

The cleavage furrow first appeared just anterior to the mouth of the opisthe at the time of separation of the halves of the dividing micronucleus. A space appeared between kinetosomes of the primary ciliary meridians, in a line around the equator of the organism (Fig. 10). The furrow deepened until the proter and opisthe were separated. Members of the genus *Tetrahymena* should be excellent organisms for testing theories of the mechanism of cleavage since they have markers, the kinetosomes, in the cortex. An examination of the pattern of infraciliature in the cortex during cleavage revealed no distortion except in the region of the cleavage furrow, where the cilia-free space appeared (Fig. 10), and where a slight torsion of the ends of the kineties next to the furrow sometimes occurred (Fig. 11). Theories which depend upon polar expansions (active or passive) of the cortex accompanying furrow formation (Swann and Mitchison, 1958) seem to be untenable for *Tetrahymena*.

Except for the absence of the micronucleus in strains GL and T-P, no differences in the relationships *between* nuclear and morphogenetic events were noted when normal cells of strain GL and mating type I, variety 1, were compared, when normal and synchronized cells of mating type I, variety 1, were compared, or when normal cells of strain T-P and mating type I, variety 1, were compared (data of Browning *et al.*, 1952).

Figure 17 is a graphic representation of the approximate time relationships of various structural and functional changes occurring in *Tetrahymena pyriformis* in a generation.

Discussion

As noted by Zeuthen (1958), there are striking associations between those biochemical changes related to energy production, and the initiation and completion of nuclear events associated with division and the process of cytoplasmic cleavage. Rate of respiration, sensitivity to anaerobiosis and to dinitrophenol and inhibitors of oxidative metabolism, nucleoside triphosphate content, and rate of P^{32} incorpora-

tion reach a maximum at the time of initiation of nuclear events. During the following period of nuclear changes, before the appearance of the cleavage furrow, the rate of respiration remains high and constant, sensitivity to anaerobiosis and to dinitrophenol and inhibitors of oxidative metabolism are at a minimum, and nucleoside triphosphate content and the rate of incorporation of P^{32} decline. When the cleavage furrow appears, sensitivity to anaerobiosis and to dinitrophenol and inhibitors of oxidative metabolism begins to rise, while nucleoside triphosphate content and the rate of P^{32} incorporation continue to decline. As cleavage proceeds, the rate of respiration begins to rise again.

The last 25% of a generation is also a period that shows the greatest fluctuations in sensitivity to extremes of temperature. Sensitivity is at a minimum during the final ⅔ of cytoplasmic cleavage. It begins to rise just before the completion of cleavage and rises gradually until the time of nuclear changes, when it rises abruptly to reach a maximum just before the onset of visible cleavage. It then drops sharply to a minimum during the first ⅓ of cleavage (Thormar, 1956). The most sensitive time coincides with the time of chromosome movement and the elongation of the micronucleus accompanying its division, just before the macronucleus begins to elongate, and at the time of final organization of the membranelles of the mouth of the opisthe. These nuclear and morphogenetic stages are characteristic of ciliates prevented from dividing by the cyclic heat treatments used to induce synchronous division (Holz *et al.*, 1957). The high sensitivity is not attributable to the effects of temperature on micronuclear division, since the amicronucleate strain GL seems to be blocked at the same point in time (Williams and Scherbaum, 1959). It is not possible at present to associate the high sensitivity of this period with any *one* of the coincident processes occurring (nuclear changes, morphogenetic changes, preparation for cytoplasmic cleavage). The cyclic heat treatments do not prevent the early stages of mitosis, amitosis or stomatogenesis, but do stop these processes at very characteristic stages and have no differential effect on them such that one progresses in time beyond the others. In normal cells from cultures which are reproducing at an exponential rate, nuclear changes and stomatogenesis also follow parallel courses, with time characteristics such that the early stages of micronuclear division, just prior to macronuclear elongation, and the late anarchic field stage and early stages of membranelle organization coincide. The fact that mitosis, macronuclear division and stomatogenesis are blocked by high temperature at characteristic stages suggests that the heat-labile biochemical system (systems) postulated by Scherbaum (1957a) and by Zeuthen (1958) is (are) necessary to the completion of all these processes, or that each process is dependent upon the others for its normal progression and goes to completion only when the others are unimpaired.

McDonald (1958), using a Feulgen microspectrophotometric method on the macronuclei of single cells, observed that DNA synthesis began after passage of about 10% of a generation, continued for approximately 45%, and ended shortly before the initiation of nuclear structural changes. In a recent preliminary note Prescott and Bors (1958) report a slightly shorter period of synthesis starting at about the same relative time and ending midway in the generation. They followed the course of synthesis by autoradiographic detection of the incorporation of tritiated thymidine. These findings are not in agreement with the earlier report

of Walker and Mitchison (1957) that a linear synthesis occurs throughout interphase. Duplication of DNA in the first half of a generation is not characteristic of all ciliates. *Euplotes eurystomus* (Fauré-Fremiet *et al.*, 1957), *Paramecium caudatum* (Walker and Mitchison, 1957), and *Paramecium aurelia* (Kimball and Barka, 1959) synthesize their DNA during the last half of a generation.

The author wishes to acknowledge with thanks the helpful suggestions of Dr. Verner Wulff and Dr. Eric Zeuthen.

Summary

The time courses of structural events occurring in a synchronous generation of *T. pyriformis,* mating type I, variety 1, were compared with one another and with the time courses of functional events in a synchronous generation, and/or in a generation of single, normal ciliates, of other strains of the species.

Note Added in Proof

Since this manuscript was accepted for publication two important relevant papers have appeared. Scherbaum *et al., J. Cell. Comp. Physiol.,* **53**: 119–138, 1959, reported that in strain GL the pattern of protein amino acids at stages in normal growth, and during the first synchronous generation of heat-treated cells, was stable. The free amino acids present at the same stages varied in their concentrations, but not in a manner that could be correlated with any of the cytological changes that occurred. Scherbaum *et al., Exp. Cell Res.,* **18**: 150–166, 1959, reported that in strain GL, during the first synchronous generation (end of heat-treatment to onset of first synchronous division), the dry weight, volume, acid-soluble phosphates, acid-insoluble heat-labile phosphates, and DNA content of the average cell increased, and thymidine incorporation into DNA could be demonstrated. In addition, circumstantial evidence from microspectrophotometric experiments, indicated a burst of DNA synthesis just prior to and during the first synchronous division. As the authors suggested, substantiation of this finding would make it unwise to apply information on DNA synthesis obtained with synchronized cells to the process in normal cells.

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ANTIGENS OF THE SEA URCHIN SPERM SURFACE[1]

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Morphological and physiological evidence indicates that the initial steps in fertilization involve the sperm and the egg surfaces. Furthermore, these steps would appear to be chemical interactions between surface substances. Beginning with the pioneer studies of F. R. Lillie (1913, 1914), the chemical relationships have been visualized as between complex molecules; and antigen-antibody systems have served as useful models (see Tyler, 1948). Most of the present information has been obtained from studies on agents extracted from sperm and eggs. Notable among these agents are the sperm isoagglutinin, fertilizin, obtained from the egg jelly layer and the egg agglutinating antifertilizin obtained from sperm. Although these and other agents have been studied extensively, their role in fertilization has not been clearly defined (see Metz, 1957a, for review). On the other hand, relatively little effort has been directed toward analysis of the intact cell surface. Contributions in this direction include Tyler's (1946) study of the effect of specific antiserum on the fertilizing capacity of sea urchin sperm. In this study non-agglutinating, univalent antibody prepared against sperm antifertilizin was found to reduce the fertilizing capacity of sea urchin sperm. In an analogous investigation on *Paramecium*, antiserum was found to block initial steps in the mating process (Metz, 1954). Extension of this line of study would seem to promise significant information for an understanding of the interaction of egg and sperm surfaces at fertilization. As a step in such an investigation it seemed desirable to map out the antigenic structure of the *Arbacia* sperm surface. Accordingly, in the present study an effort was made to determine the number of sperm surface antigens and their distribution with respect to the morphology of the sperm, and to examine for sperm surface antigens that do not appear in sea water extracts.

Studies on the antigenic structure of sperm are found in publications extending over a period of 60 years. However, most of such studies were not directed primarily toward an understanding of the fertilization process. More recently Mudd and Mudd (1929), Henle (1938), Snell (1944), Smith (1949) and Pernot (1957) among others have demonstrated species, strain and tissue specificities. Certain of these studies, notably those of Henle *et al.* (1938) and Pernot (1957), are also concerned with the number and distribution of sperm antigens. The former study showed three agglutinogens on bull sperm, one confined to the tails, one restricted to heads and the third common to both. Pernot's (1957) agar diffusion and immunoelectrophoretic study of guinea pig material revealed eleven antigens in

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the seminal plasma and seven in alkaline sperm tail extracts. Six antigens were common to both materials. Pernot also presents some evidence for an antigen common to both heads and tails, and an experiment suggesting another antigen restricted to the guinea pig sperm tail. It remains to be shown which if any of the sperm antigens in Pernot's extracts are components of the cell surface. Finally, Dallam and Thomas (1953) have isolated lipoprotein from sperm heads of several mammalian species. Antisera prepared against such lipoprotein agglutinated sperm.

These studies concern mammalian material. Among echinoderms extensive serological investigations of the egg have been made by Tyler and Brookbank (1956a, 1956b), and the Perlmanns (Perlmann, 1957; Perlmann and Perlmann, 1957a, 1957b; see Runnström *et al.*, 1959, for review). Studies of the sperm include a brief reference by Perlmann (1957) and accounts by Tyler and O'Melveny (1941) and Tyler (1946, 1949). In the last report Tyler (1949) lists cross-agglutination reactions between antiserum and sperm of several species and demonstrates a minimum of two antigens on the *Strongylocentrotus purpuratus* sperm surface.

Material and Methods

Seven echinoderms were employed in the study. These include *Arbacia punctulata, Lytechinus variegatus, Mellita quinquiesperforata* and *Plagiobrissus grandis* from the vicinity of the Florida State University Marine Laboratory at Alligator Point, Florida, and *Arbacia punctulata, Echinarachnius parma* and *Asterias forbesii* from Woods Hole, Mass.

Gametes were obtained from *Arbacia* by electrical stimulation (see Harvey, 1956) or by excising the gonads. Treatment with isotonic KCl was avoided since this method causes release of the antigen-containing dermal secretion (Metz, 1959). Gametes were obtained from *Lytechinus, Mellita, Echinarachnius* and *Plagiobrissus* by treating the gonads with isotonic KCl.

Sperm and seminal plasma were separated by low speed centrifugation. The sperm was re-suspended once in sea water and centrifuged again. The supernatant was discarded and the settled sperm was made up as a 25% suspension by addition of three volumes of sea water. This stock suspension was used for injections and absorptions. For spot plate agglutination tests a 1% sperm suspension was prepared by diluting the stock suspensions to $\frac{1}{25}$ with sea water.

Injections. To obtain antisera five rabbits were injected with *Arbacia* sperm (25% washed sperm in sea water) or sperm extracts, three rabbits were injected with *Lytechinus,* one with *Echinarachnius* and three with *Asterias* sperm. The immunizing antigens were administered through intravenous, intraperitoneal and subscapular injections. In the last instance the antigen was injected as an emulsion in Freunds' adjuvant (Difco). This yielded the antisera with the highest sperm agglutinin titers. Most of the rabbits received two or three courses of injections.

Although anti-*Arbacia* sera from several rabbits were examined with uniform results only experiments with antiserum from the hyper-immune rabbit "#33" are recorded here. This rabbit was injected with antigen in Freunds' adjuvant. The animal was bled two weeks subsequent to the injection. The immune serum obtained regularly agglutinated sperm to dilutions of 2^{-8} to 2^{-10}. No sera were pooled.

Tests, assays and absorptions. The sperm agglutination tests were performed by mixing one volume (usually one drop) of 1% sperm suspension with two volumes (2 drops or 0.1 ml.) of serum in depression slides. The salt concentration of sera was adjusted to that of sea water by dialysis or addition of an equal volume of 1.73 × sea water. Reactions were recorded after the mixtures had remained for three hours at room temperature. Parallel tests with control (preinjection) serum were performed in all experiments to control for nonspecific reactions.

The agglutination of *Arbacia* sperm with antiserum gave a regular pattern. When diluted serially in sea water the antiserum gave titers with sharp, reproducible end points. However, the morphology of the agglutinates varies upon dilution of the antiserum. Antiserum at dilutions to 2^{-5} yielded net-like agglutinates involving head-to-head, head-to-tail and tail-to-tail agglutination. Agglutination at higher antiserum dilutions was head-to-head, the sperm was motile and the agglutinates were "bouquet-like" in structure. Sperm in such dilute antisera fertilized eggs and became more active on addition of fertilizin. This last effect was associated with a breakdown of the antiserum agglutinates.

The absorptions were done with varying amounts of material. Absorbing antigen and antibody were usually allowed to react for three hours at room temperature. Absorption over a longer time did not result in lower titers.

Isolation of sperm tails and heads. Isolated sperm tails and heads were used in absorption and agglutination experiments. In order to separate sperm tails and heads washed, living or formalin-killed sperm (25% suspension) was subjected to the shaking action of the Mickle disintegrator for 20 minutes at room temperature. The resulting material was then centrifuged at low speed at 4° C. to sediment the sperm heads. Suspensions of tails and of heads were washed when necessary by high and low speed centrifugation, respectively, at 4° C.

Results

In this investigation primary interest centers about the sperm "surface" as opposed to "subsurface" or interior material with antigenic properties. Although the sperm surface may be difficult to define or delimit at the molecular level, present requirements are satisfied by an operational definition. According to this definition, sperm surface antigens (specific combining sites) are those antigens so situated that their combination with antibody under appropriate conditions results in sperm agglutination. In any event information concerning the role of the sperm surface in fertilization is the ultimate objective here and antigenic groups that are available for participation in the agglutination reaction should be sufficiently accessible for interaction with the egg surface in the initial stages of fertilization.

In the experiments described below some attention is given to the antigenic composition of sperm extracts. It is clear that the "surface" vs. "subsurface" origin of soluble antigens in sperm extracts is not readily established. For example, a single antigenic group might be readily extracted from the cell "interior" and at the same time exist in bound, unextractable and insoluble form at the cell "surface." However, removal of sperm agglutinating antibodies from antisera by absorption with sperm extracts serves to identify and separate antibodies against sperm surface antigens irrespective of the origin of the absorbing antigens.

Such absorbed sera in turn can serve to separate and identify sperm surface antigens. The experiments described below should be considered in this conceptual framework.

A) *Identification of sperm surface antigens by interspecific reactions*

In order to identify sperm surface antigens, cross-absorption tests were performed in interspecific, agglutinating combinations of sperm and antisera. Six foreign sperms were tested for cross-reaction against antisera prepared against sperm of four of the species. With the exception of the combination *Mellita* sperm

TABLE I

Sperm agglutinating action of antisera before and after absorption with sperm of various species

Sera		Immune sera Tested with					
Prepared against sperm	Abs. with sperm	A*	L	E	M	P	Asterias
A*	—	+	+	+	0	+	0
A	A	0	0				
A	L	+	0	0			
A	E	+	+	0			
A	E + L	+	0				
L	—	+	+	+	+(weak)	+	+(weak)
L	L	0	0				
L	A	0	+	0			
L	E	+	+	0			
L	E + A	0	+	0			
E	—	+	+	+	+		+
E	L	0	0	+			
E	A	0	+				
E	A + L	0	0	?			
Asterias	—	0	0		0		+

*A = *Arbacia;* E = *Echinarachnius;* L = *Lytechinus;* M = *Mellita;* P = *Plagiobrissus.*
Control (pre-injection) sera failed to agglutinate sperm in parallel tests to those in the table.

vs. *Arbacia* antiserum, cross-reactions were found among all echinoid combinations tested. These relations are summarized in Table I.

Certain of these cross-reacting combinations were used in cross-absorption experiments. These included the series *Arbacia, Lytechinus* and *Echinarachnius* as seen in Table I. Absorption with the homologous sperm rendered antisera non-agglutinating for the cross-reacting heterologous species. In every case complete absorption with an heterologous sperm yielded a serum which still agglutinated the homologous species. Furthermore, in most cases such absorbed sera agglutinated the other heterologous species as well. Thus anti-*Arbacia* serum absorbed with *Echinarachnius* sperm still agglutinated *Lytechinus* and *Arbacia* sperm.

Evidently, then, *Lytechinus* and *Arbacia* sperm possess surface antigens lacking on *Echinarachnius*. A further absorption of the *Echinarachnius* sperm absorbed anti-*Arbacia* serum with *Lytechinus* sperm yielded a serum which failed to agglutinate *Lytechinus* but still agglutinated *Arbacia* sperm. Accordingly, it is concluded that the Arbacia sperm surface possesses at least three antigens or antigenic complexes, one specific for *Arbacia*, a second shared with *Lytechinus* and a third shared with *Echinarachnius*. It is of some interest that *Lytechinus* sperm absorbs all *Echinarachnius* sperm agglutinins from anti-*Arbacia* sperm serum. This indicates that all the "*Arbacia* antigens" present on the *Echinarachnius* sperm surface are also present on the *Lytechinus* sperm. Comparable results were obtained in absorption experiments using anti-*Lytechinus* sperm serum (Table I).

B) *Sperm surface antigens in sperm extracts*

In the previous section the *Arbacia* sperm surface was shown to possess at least three antigens or antigenic complexes. In view of the possible role of insoluble as well as soluble sperm surface substances in fertilization (see Metz, 1957a), it seemed of interest to examine sperm extracts for soluble antigens in common with sperm surface antigens. This matter is of special interest because it concerns the question whether the antifertilizin extracts from sperm contain the full complement of sperm surface antigens. In one series of experiments soluble antigen extracts were prepared by freeze-thawing *Arbacia* sperm. This is one method for preparing sperm antifertilizin (Tyler, 1939). All extracts were subject to low speed centrifugation (3000 × g for 20 minutes) to remove fragments of the sperm surface. Certain preparations were also subjected to high speed centrifugation (26,000 × g) to remove the larger sub-cellular particulates. Constant amounts of anti-*Arbacia* sperm serum were then absorbed with increasing amounts of the sperm extracts. The absorbed sera were then assayed for sperm agglutinating action. As seen in Figures 1a, 3a, 4a, and 6a, increasing amounts of sperm extract lowered the sperm agglutinin titer until a constant value was reached. Beyond this point increasing amounts of extract fail to affect the sperm agglutinating action. The extracts failed to remove all of the sperm agglutinins (seven experiments). Accordingly, it appears that the extracts do not contain all of the sperm surface antigens. Some of these evidently are not extractible in sea water by freeze-thawing and do not appear as components in extracted "antifertilizin." Most of these antigens are heat-labile. If the frozen-thawed extract is heated a precipitate begins to form at 55° C. and after further heating for one minute at 100° C. the supernatant has reduced antifertilizin activity and lowers the sperm agglutinating titer of antiserum only one dilution step (compare Figure 1, a and b).

In view of the antibody absorbing action of extracts prepared by freeze-thawing sperm, it seemed of interest to examine extracts prepared by other methods for antigens in common with those present on the intact sperm surface. Accordingly, extracts were prepared by heating (100° C. for one minute) followed by centrifugation, by disruption in the Mickle disintegrator, and by acid extraction.

As seen in Figure 2a (typical of two experiments), extracts prepared by heating had little if any effect on the sperm agglutinating action of anti-sperm serum. Evidently, then, heat extraction yields few if any sperm surface antigens. However, antigenic material is present in extracts prepared by heating, for such extracts

produce one precipitin band in agar when diffused against antiserum (to be published). Possibly this is sub-surface material. If so, it suggests that the antifertilizin (egg jelly precipitating and fertilizin neutralizing) activity of extracts prepared by heating (Frank, 1939) is not due to sperm surface material. Finally, as seen above, frozen-thawed extracts contain insignificant amounts of surface antigenic material after heating to 100° C. Extracts prepared by action of the Mickle disintegrator also neutralized the sperm agglutinating action of anti-whole sperm serum. As seen in Figure 2c (two experiments), such absorption reduced the agglutinin titer to a constant level, but failed to remove all agglutinins.

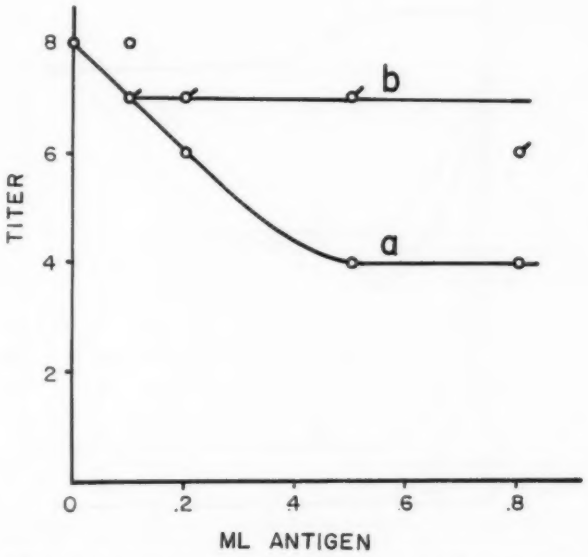

FIGURE 1. Effect of absorption with increasing amounts of antigen on sperm agglutinating action of anti-*Arbacia* sperm serum. Curve (a): Antiserum absorbed with frozen-thawed sperm extract, tested on *Arbacia* sperm. Curve (b): Antiserum absorbed with heated (100° C., one minute) frozen-thawed sperm extract, tested on *Arbacia* sperm. Ordinate: Titer $= -\log_2$ of highest antiserum dilution giving sperm agglutination. Abscissa: ml. antigen added to 0.5 ml. antiserum.

Acid extraction of sperm also yields antifertilizin (Tyler and O'Melveny, 1941). In one experiment anti-whole sperm serum was absorbed with increasing amounts of neutralized pH 3.0 sperm extract. Figure 2b again shows reduction of the agglutination titer to a constant level. In an additional comparative experiment sperm suspensions (12.5%) were extracted at pH 3.2, 7.2 and 9.0 for six hours. The extracts were subsequently tested for antibody absorbing action. The pH 7.2 ("aged sperm") extract lowered the sperm agglutination titer a maximum of one-half (one-fold) whereas the acid and alkaline supernatants lowered the titer of the antiserum a maximum of ⅛ (three-fold). Thus, acid and alkaline extracts

of sperm, like frozen-thawed and Mickle extracts, contain some but not all of the antigenic groups present on the sperm surface.

Relation of antigens in sperm extracts to fertilizin. In view of the fact that the soluble antigen preparations contain antifertilizin, it seemed of interest to examine the relations of such extracts and of anti-sperm serum to fertilizin. Specifically, it appeared of interest to test for action of fertilizin on the antiserum neutralizing action of soluble sperm antigen extracts (antifertilizin). To test for neutralizing

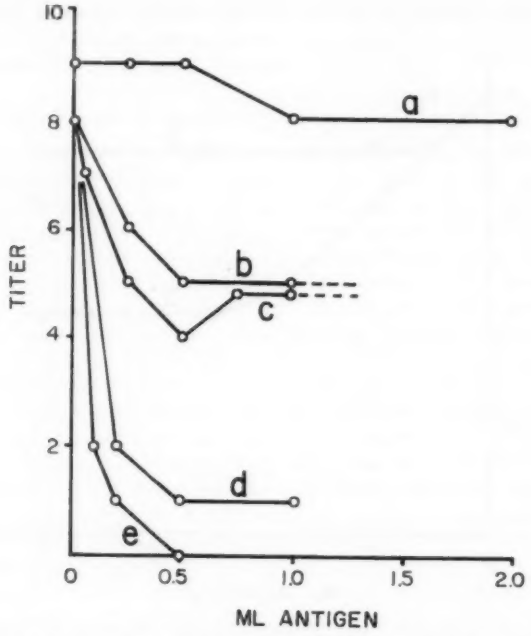

FIGURE 2. Effect of absorption with increasing amounts of antigen on sperm agglutinating action of anti-*Arbacia* sperm serum. Curve (a): Absorbing antigen prepared by heating (100° C., one minute) sperm. Curve (b): Absorbing antigen prepared by extracting sperm in pH 3.0 sea water. Curve (c): Absorbing antigen prepared by Mickle disintegration of sperm. Curve (d): Absorbing antigen: isolated sperm heads. Curve (e): Absorbing antigen: whole intact sperm. Absorbing antigen for curves (a), (b) and (c) centrifuged 3000 × g. Each curve represents a different experiment. Ordinate and abscissa as in Figure 1.

action, fertilizin and antiserum were mixed in varying proportions and the sperm agglutinin titers determined. The experiment is complicated by the fact that fertilizin and antiserum both agglutinate sperm. To avoid confusion from the action of the two different agglutinins, the sperm was examined for agglutination after sufficient time (three hours) to permit reversal of any agglutinating action of fertilizin (see methods section). As seen in Figure 3b the sperm agglutinin titer of the antiserum was not reduced by fertilizin. It appears, then, that the

sperm surface and fertilizin do not have antigenic combining sites in common which are essential for sperm agglutination.

On the other hand, the combining sites of the soluble antigens in frozen-thawed extracts evidently are blocked by fertilizin. This is again seen in Figure 3, curve c. In this experiment decreasing amounts of fertilizin were mixed with increasing amounts of the sperm surface extract (antifertilizin). The mixtures were subsequently used to absorb the anti-sperm serum. When the extract-to-fertilizin ratio equalled 0.143 (0.25 ml. extract) the mixture failed to affect the

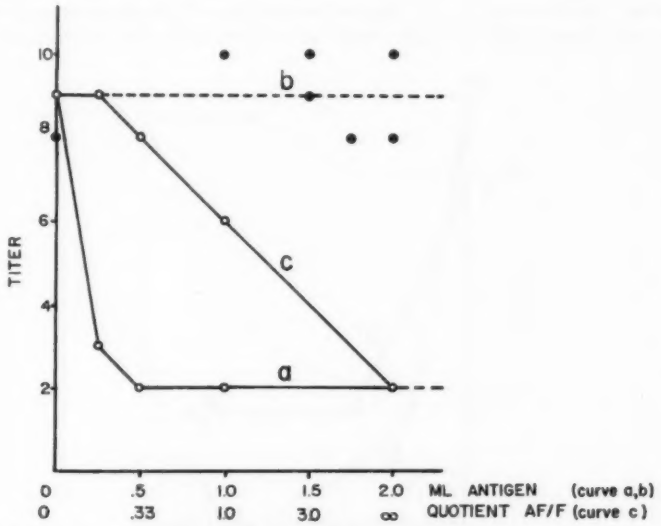

FIGURE 3. Effect of absorption with increasing amounts of antigen on the sperm agglutinating action of anti-*Arbacia* sperm serum. Curve (a): Serum absorbed with sperm extract (antifertilizin) prepared by freeze-thawing sperm and centrifuging at 3000 × g for 20 minutes. Curve (b): Antiserum absorbed with fertilizin. Curve (c): Antiserum absorbed with anti-fertilizin-fertilizin mixtures. Ordinate: Titer = $-\log_e$ of highest antiserum dilution giving sperm agglutination. Abscissa for (a) and (b): ml. antigen added to 0.5 ml. antiserum. For (c): Quotient antifertilizin/fertilizin/0.5 ml. antiserum. Two ml. fertilizin and no anti-fertilizin at quotient = 0. The same fertilizin preparation was used for curve b and c. Absolute amounts of antifertilizin, antisera and total volumes in curves (a) and (c) are identical along the abscissa.

sperm agglutinin titer of the anti-sperm serum, whereas the same amount of anti-fertilizin alone reduced the agglutinin titer six-fold. Increase in the proportion of antifertilizin resulted in mixtures with antiserum neutralizing action. This result shows that fertilizin neutralizes the ability of the frozen-thawed extract to absorb sperm agglutinins from the anti-sperm sera.

Distribution of "soluble" surface antigens. Data in the previous section show that extraction of sperm yields solutions of soluble sperm antigens and that these neutralize some of the antibodies (agglutinins) for the sperm surface. On the

other hand the extracts fail to neutralize all of the sperm agglutinating antibodies. Accordingly, the extraction procedures fail to remove some "insoluble" antigenic material from the sperm. Although the antigens in the preparation may not be extracted from the sperm surface they are antigenically related to, and therefore serve to identify, surface antigens. In an attempt to localize these "soluble antigens" on the sperm surface, antisera were treated with extracts prepared by freeze-thawing (antifertilizin) and then tested for agglutinating action on suspensions of isolated sperm heads, tails and intact sperm. As seen in Figure 4 such extracts completely neutralized the agglutinins for sperm tails. Evidently, then, the sperm tail surface possesses only soluble antigen. In this connection it is of interest that agar diffusion precipitin tests show only a single band when frozen-thawed extract of isolated

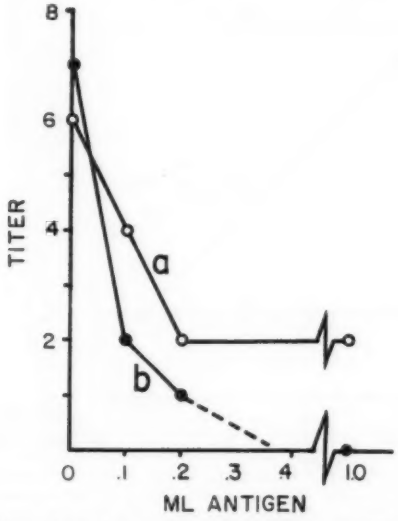

FIGURE 4. Agglutination of whole sperm and isolated sperm tails with anti-*Arbacia* whole sperm serum absorbed with frozen-thawed whole sperm extract (antifertilizin). Curve (a): Agglutination of whole sperm. Curve (b): Agglutination of isolated tails. Ordinate and abscissa as in Figure 1.

sperm tails is diffused against anti-whole sperm serum. This single band merges with one of four produced by whole sperm extract (to be published). These observations show that the insoluble antigens are confined to the sperm head. This view is confirmed by examination for agglutination of whole sperm and heads by the absorbed sera. As seen previously (Fig. 3a) the agglutinin titer for whole sperm is reduced to a constant level by absorption with frozen-thawed extracts. On the other hand, such extracts had no apparent effect on the ability of anti-sperm serum to agglutinate isolated sperm heads. Evidently, the surfaces of the isolated sperm heads and the frozen-thawed extracts do not have antigens in common and the sperm heads, prepared by treatment in the Mickle disintegrator, lack (have lost?) soluble surface antigenic material.

Regional localization of sperm surface antigens. In a previous section it was shown that whole sperm extract absorbed the agglutinins for isolated *Arbacia* sperm tails but failed to absorb all agglutinins for isolated sperm heads. This result indicates that the sperm tail antigens are soluble, whereas some or all of the antigens on the sperm head surface are insoluble. It seemed of interest to extend this study to a more direct analysis of differences between sperm heads and tails. To achieve this the method used by Henle *et al.* (1938) for bull sperm was employed. *Arbacia* sperm heads and tails were isolated by treatment in the Mickle disintegrator fol-

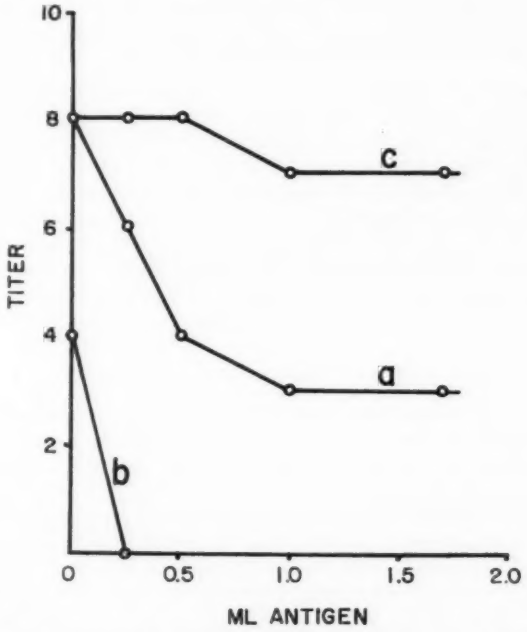

FIGURE 5. Sperm agglutinating action of anti-*Arbacia* whole sperm serum after absorption with sperm tails isolated from formalin-killed sperm. Curve (a): Tested on living *Arbacia* sperm. Curve (b): Tested on sperm tails isolated from formalin-killed sperm. Curve (c): Tested on sperm heads isolated from formalin-killed sperm. Ordinate and abscissa as in Figure 1.

lowed by differential centrifugation. The suspensions of isolated heads and tails were then used to absorb anti-whole sperm serum. Preliminary experiments showed that isolated tails always reduce the sperm agglutination titer in absorption experiments. Isolated sperm heads, however, showed varying results and require further study. The inconsistencies may be related to the presence of very readily soluble sperm surface antigens which appear in washings of the sperm.

Unfortunately, treatment of living sperm in the Mickle disintegrator resulted in considerable fragmentation of the sperm tails. This was especially true in the

preparation of large amounts of tails for absorption experiments. To facilitate isolation of unbroken tails sperm were first fixed in 1% formalin, washed in saline and the tails shaken from the remainder of the sperm in the Mickle disintegrator.

In using formalin-fixed sperm it is assumed that the formalin treatment does not alter the antigenic structure of the sperm surface. This assumption is valid to the extent that both living and formalin-killed whole sperm and sperm fragments agglutinated to the same titer with anti-sperm serum and that living and formalin-fixed material showed parallel absorbing action in preliminary experiments. For

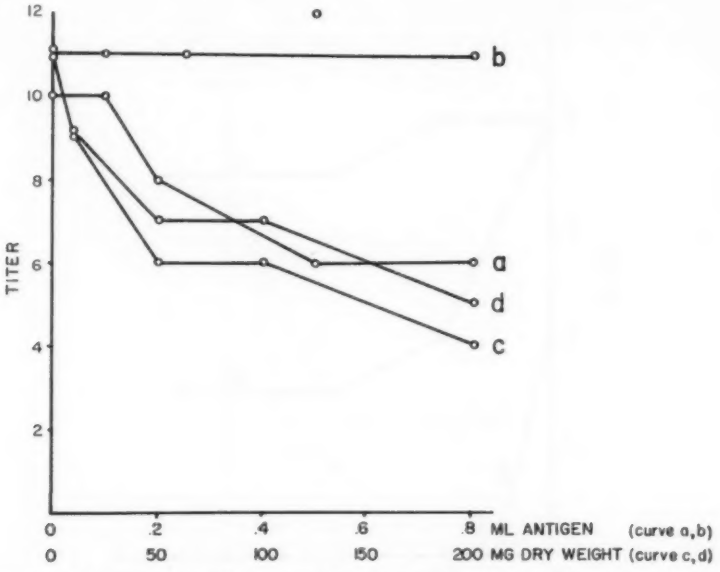

FIGURE 6. Sperm agglutinating action of anti-*Arbacia* whole sperm serum absorbed with *Arbacia* antifertilizin and sperm "ghosts." Curve (a) and (b): Absorbing antigen, frozen-thawed sperm extract. (a): Tested on *Arbacia* sperm. (b): Tested on *Lytechinus* sperm. Abscissa: ml. antigen added to 0.2 ml. antiserum. Curve (c) and (d): Absorbing antigen, frozen-dried "ghosts." (c): Tested on *Arbacia* sperm. (d): Tested on *Lytechinus* sperm. Abscissa: mg. dry weight. Ordinates for (a), (b), (c) and (d): Titer $= -\log_2$ of highest antiserum dilution giving sperm agglutination.

a final experiment washed sperm was fixed in formalin (1%), washed and treated for 30 minutes in the Mickle disintegrator. The tails were then isolated from the intact sperm and heads by centrifugation. Finally, the isolated tails were used in varying amounts to absorb the anti-whole sperm serum. This absorbed serum failed to agglutinate tails (Fig. 5b). However, it did agglutinate isolated sperm heads (Fig. 5c) and living whole sperm (Fig. 5a), but in both instances the absorbed serum showed lower titers than the unabsorbed serum. Evidently, then, the sperm heads possess a head specific antigen or antigens which are not present on sperm tails. The reduction in titer of sperm head agglutination by absorption

with tails indicates that some tail antigenic material is also present of the sperm heads.

Analysis of insoluble sperm surface antigens. In order to confirm the presence of water-insoluble sperm surface antigens it seemed of interest to attempt to absorb sera with sperm from which the soluble sperm antigens had been removed. Sperm "ghosts" were prepared by freezing 25% sperm, removing the supernatant, re-freezing the re-suspended residue and finally washing the insoluble material five times in sea water. As seen in Figure 6a, the initial extract of soluble antigens (antifertilizin) lowered the sperm agglutinin titer four-fold whereas the extracted "ghosts" when used in excess lowered the titer seven-fold (Fig. 6c). Evidently, then, the extracted "ghosts" possess insoluble antigens that are not present in the soluble fraction.

Furthermore, it is of interest that these *Arbacia* "ghosts" also lowered the sperm agglutinin titer for *Lytechinus* sperm (curve 6d), whereas the absorption with frozen-thawed extract (Fig. 6b) did not affect the titer for *Lytechinus*. It remains for future experiments to determine if all the antigens common to the two species are insoluble, and if the ones in the extracts are the specific antigens.

Presence of sperm surface components in body fluid and egg extract. Results from several experiments show that neither body fluid from male or female sea urchins nor egg extract contains sperm surface components involved in antiserum agglutination. Absorption of whole sperm antiserum with these extracts did not lower, or lowered only one step, the titer for sperm agglutination.

Discussion

In view of the striking morphology of the spermatozoan, the multiple antigen structure of cell surface generally (*e.g.* erythrocytes) and the presence of at least three distinct surface antigens on bull sperm (Henle *et al.*, 1938), and two on *Strongylocentrotus* sperm (Tyler, 1949), it is not surprising to find several antigens on the *Arbacia* sperm surface. The distinct head and tail antigens found on *Arbacia* sperm conform to the pattern found for bull sperm. It seems likely that tests of a wider range of heterologous species would reveal more cross-agglutination reactions between foreign sperm (*e.g.* Tyler, 1949) and the anti-*Arbacia* sperm serum. Absorption of the antiserum with such foreign sperm might reveal more antigens in addition to the three so far demonstrated on the *Arbacia* sperm surface. Furthermore, a more detailed analysis with such absorbed sera, such as testing for agglutinating action on isolated sperm heads and tails, should localize the individual antigens or antigenic complexes.

The number and distribution of the sperm surface antigens is not without interest, for this information contributes to the knowledge of sperm surface architecture. Furthermore, such antigens could be used as additional points of attack in studies on the role of the sperm surface in fertilization. However, the relationship of the sperm surface antigens to antigens present in sperm extracts appears to be of more immediate interest.

Extracts of sea urchin sperm prepared by heating (Frank, 1939), freeze-thawing (Tyler, 1939) and acid extraction (Tyler and O'Melveny, 1941) contain an agent or agents called antifertilizin, which neutralizes the sperm agglutinating action of fertilizin, agglutinates eggs and precipitates the egg jelly. This antifertilizin in

sperm extracts is assumed to come from the sperm surface and to include the specific sperm surface combining sites with which fertilizin reacts when it reversibly agglutinates sperm.

In support of this view Tyler (1949) extracted antifertilizin from the *Strongylocentrotus* sperm by acid treatment. Subsequent examination of the cells with the electron microscope revealed a partial breakdown of the sperm head surface, suggesting this as the site of origin of the antifertilizin. However, the situation is probably more complicated than this, for as seen in the present study absorption of anti-whole sperm serum with sperm extracts (antifertilizin) removes tail agglutinins but has little effect on the head agglutinins. This suggests that the antifertilizin may be present in large amounts on the sperm tail.

Furthermore, agar gel precipitin tests show that the antifertilizin extracts prepared by freeze-thawing regularly contain three to four antigens (to be published). The surface vs. sub-surface origin of these antigens is not immediately clear. However, the fact that this mixture of antigens will remove some sperm agglutinins from anti-sperm serum suggests that at least one of the antigens is of sperm surface origin. But even this experiment does not exclude the possibility that the absorbing antigen(s) possesses a combining group in common with the sperm surface but is actually extracted from the interior of the sperm.

In further attempts to identify these antigens the antigen neutralizing action of fertilizin was examined. Following treatment with fertilizin, antifertilizin failed to lower the sperm agglutination titer of the antiserum. Clearly, then, fertilizin can neutralize all the sperm surface antigens in the frozen-thawed extract. The fertilizin may be highly specific in action and neutralize only one of the four antigens. If this is the case, it follows that the extract prepared by freeze-thawing contains only one antigen in common with the sperm surface. In any event, the antigen or antigens involved appear to be related mainly to the antigens of the sperm tail (see above).

Studies on the role of specific egg and sperm substances in metazoan fertilization have been concerned mainly with soluble agents. It is of some interest, then, to find that extracts of sperm do not contain all of the surface antigens. Evidently, some antigens are either destroyed by the extraction procedure or remain in the insoluble residue. The second alternative seems the more likely in view of the nature of the extraction procedure. Indeed the absorption experiments with sperm "ghosts" show that insoluble antigens survive the extraction procedure. The role in fertilization of such insoluble sperm surface antigens remains to be investigated. The specific combining groups on the sperm surface that function in the antiserum agglutination reaction may not be identical with molecular configurations that function in fertilization. Nevertheless it seems likely that such material may form a part of, or be spatially related to, molecular surface patterns that do function in the union of sperm and egg. This apparently is the case in *Paramecium* (Metz, 1954).

Consideration here has been given only to the sperm head and tail. A more detailed study should take into account the subdivisions of these structures, such as the axial tip of the tail, and the midpiece and acrosome of the head. Furthermore, especial attention should be given to comparison of the antigens of the sperm before and after acrosomal filament discharge, for at least in some forms (*e.g.* starfish) activation of the egg results from interaction of the egg surface with the tip

of the acrosomal filament (see Metz, 1957b; Colwin and Colwin, 1957; Collier, 1959; Dan, 1956).

Summary

1. Sperm surface antigens are defined as those antigens which are detected by sperm agglutinating action of antisera.

2. Interspecific sperm agglutination tests show one or more sperm surface antigens common to all of the five echinoids tested except for a single combination.

3. The *Arbacia* sperm surface possesses a minimum of three antigens or antigenic complexes in common with other species. One of these is shared with *Lytechinus* sperm, a second with both *Lytechinus* and *Echinarachnius* and a third is not present on sperm of these species.

4. *Arbacia* sperm extracts prepared by freeze-thawing, Mickle disintegration or pH 3 treatment all contain "soluble" antigens in common with the sperm surface. The *Arbacia* sperm surface also possesses "insoluble" antigens which do not appear in the above extracts.

5. The "soluble sperm surface" antigen(s) in extracts is neutralized by fertilizin from *Arbacia* eggs.

6. It is present on the sperm tail surface and possibly to some extent on the sperm head.

7. The insoluble sperm surface antigen(s) is confined to the sperm head.

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THE RELEASE OF EFFERENT NERVE ACTIVITY IN THE ROACH, PERIPLANETA AMERICANA, BY EXTRACTS OF THE CORPUS CARDIACUM[1]

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Several attempts have been made to correlate patterns of endogenous activity as observed in isolated portions of invertebrate nervous systems with the normal behavior of the intact animal (von Holst, 1934; Roeder, 1955; and Harker, 1956 and 1958). Roeder, Tozian and Weiant (1960) found that decapitation of roaches or of mantids markedly increases the level of endogenous activity in the efferent nerves from the cercal ganglion which innervate the phallic musculature and in the efferent nerves from the metathoracic ganglion which innervate the thoracic musculature. As the activity in the phallic nerves increases, rhythmic bursts of nerve spikes are seen. These motor bursts on the several nerve branches leading to the phallomeres are not obviously coordinated, but they result in ordered, rhythmic movements of the genitalia. The behavioral significance of this endogenous nerve activity is suggested by the observation that in male mantids the copulating reflexes appear with great intensity following decapitation. Indeed, under natural conditions, the male is often beheaded by the female before he mates with her (Roeder, 1935). Control of this sexual behavior and of the efferent nerve activity in the nerves to the phallomeres and to the thoracic musculature appears to be effected through the agency of an inhibiting center in the subesophageal ganglion.

In most of these experiments (Roeder, Tozian and Weiant, 1960) there was a time lag of five to fifteen minutes between the removal of the head and the appearance of bursts of spikes in the efferent nerves. This time lag might result from a decreasing discharge of severed inhibitor neurones, augmented by injury potentials. On the other hand, it might represent the time necessary for the inactivation or removal of an inhibiting neurohormone. Such a material, if present, could be transported by proximo-distal flow down axons within the nerve cord to the site of its action.

A variety of substances are to be found in roaches which affect their behavior and some of these may be produced within the central nervous system itself (Beament, 1958; Colhoun, 1958, 1959; Smyth, 1959; and Sternberg and Kearns, 1952). The work of Özbas and Hodgson (1958) showed that the injection of corpus cardiacum extracts produced stereotyped behavior in roaches and that these extracts had a pronounced inhibitory effect on the activity in isolated roach abdominal nerve cords. Their results led us to attempt to block the inhibitory influences

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impinging on the efferent nerve cells in the cercal and metathoracic ganglia by the use of similar extracts.

Materials and Methods

Most of the observations of activity in efferent nerves to the phallomeres and in the connectives of the abdominal nerve cord were made using male *Periplaneta americana* as test animals. For these experiments the animals' wings and legs were removed and the bodies were pinned dorsal side down on a cork. Most of the abdominal contents were removed. Nerve IX or nerve X from the cercal ganglion (Fig. 1, E^2) was picked up on a small tapered silver wire hook electrode and allowed partially to dry. In many experiments afferent impulses in the nerve and the muscle potentials resulting from the efferent activity were eliminated by cutting the nerve distal to the recording electrode. The indifferent electrode was a chlorided silver wire touching the inner abdominal wall. Simultaneous recordings of nerve activity in the connectives of the abdominal cord were made by raising a connective on a pair of silver hook electrodes (Fig. 1, E^1). The input from the phallic nerve fibers was amplified with a Grass P6 preamplifier and observed on a Dumont 304A oscilloscope. The amplified signals were also recorded on one channel of a Grass Polygraph inkwriter using a 5P3 integrator preamplifier. The activity from the abdominal connectives was amplified by a 5P3 preamplifier and appeared on the other channel of the inkwriter. The two inputs could be transposed at any time so that visual observations or photographs could be made of either the nerve impulses from the cord or of those from the phallic efferent nerves. Meanwhile, a continuous record of the integrated activity from both sources appeared on the inkwriter.

Endogenous efferent nerve activity in the thoracic region was sampled from an electrode pair placed under nerve 3 (Fig. 1, E^3) from the left side of the metathoracic ganglion. The method has been described previously (Weiant, 1958). All other nerves arising from the thoracic ganglia were severed, eliminating all sensory input from these segments. The longitudinal connectives between the thoracic ganglia were left intact, as were the connections of the thoracic cord with the head ganglia and abdominal ganglia. Peripheral connections of extra-thoracic regions of the nervous system were also left intact. Activity of the internuncial fibers within the thoracic connectives was monitored simultaneously with that of nerve 3 by placing a pair of electrodes under the thoracic connectives above and below the prothoracic ganglion (Fig. 1, E^4).

Many of the same extract samples were tested for their action both on the phallic motor nerves and on the thoracic efferent fibers. In experiments with both of these preparations, injections into the head capsule of 0.01 to 0.02 ml. of extract were made as close to the subesophageal ganglion as possible. In some tests with the thoracic preparation a drop of extract was applied directly on the metathoracic ganglion; in other experiments using the abdominal preparation, several drops of extract were used to bathe the last three abdominal ganglia with their connectives. Some tests were conducted on *Periplaneta* from which the corpora cardiaca had previously been removed.

Extracts were made from the corpora cardiaca of the following species of roaches: *Blaberus craniifer, Blaberus giganteus, Periplaneta americana, Leucophaea*

maderae, Byrsotria fumigata, and *Diploptera punctata.* Extracts were from the glands of adult animals except in one case in which an extract was made from the corpora cardiaca of penultimate stage nymphs of *Periplaneta.*

The paired corpora cardiaca were excised according to the technique of Özbas and Hodgson (1958) and placed on the end of a small glass pestle. The glands were removed as quickly as possible after the donor roaches had been caught in order to minimize the loss of active material due to excitation of the roach (Hodgson, personal communication). When the requisite number of corpora cardiaca had been collected, they were triturated in a small mortar. Either Hoyle's or Pringle's insect saline was added from a hypodermic syringe to dilute the extract to the concentration desired. Pringle's proved to be the more satisfactory saline for maintaining the nerve activity in the preparations.

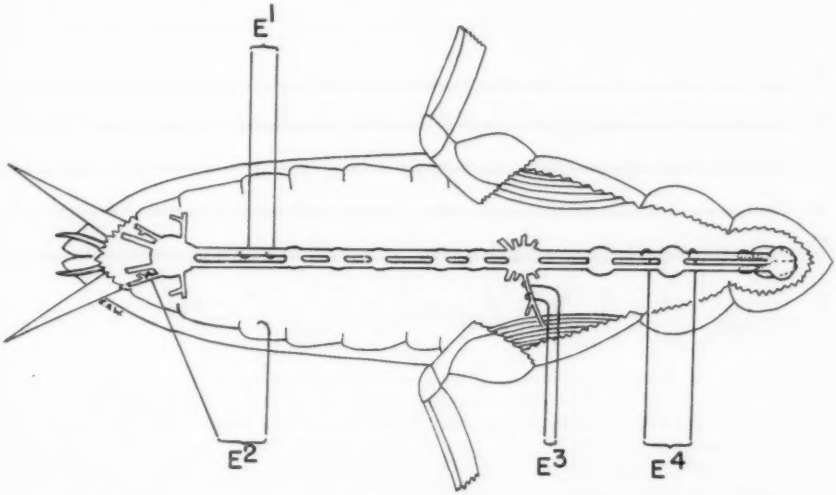

FIGURE 1. Diagram of the central nervous system of *Periplaneta americana* showing placement of electrodes.

In many of the experiments the mortar containing the extract was heated at 95° to 100° C. for five minutes. A coagulum formed in the bottom of the mortar, leaving a fairly clear solution which was used for the tests. Longer exposures to heat seemed to impair the activity of the extract, but a brief period of heating left the potency unchanged. Unheated extracts lost their activity somewhat after four to five hours at room temperature or after about ten hours at 12° to 14° C. Heating approximately doubled the time that the extracts would remain active.

Four experiments were conducted with extracts which were quick-frozen on blocks of dry ice and kept frozen for periods of twenty-four hours to a week. Two of these extracts were heated before being frozen. None of the quick-frozen extracts showed any loss of activity. This seems a promising method for preserving such material.

Results

Efferent nerve activity of the last abdominal ganglion. The effects of decapitation or of nerve cord transection on efferent fiber activity of the last abdominal ganglion have been described in detail elsewhere (Roeder, Tozian and Weiant, 1960). In a roach with the nervous system intact, spike potentials are observed in only a few efferent fibers of the nerves supplying the phallic musculature. In those fibers which are active, spikes recur regularly and at a relatively low frequency. If the animal is decapitated, there is a brief injury discharge followed by a return to the previous level of activity. After five to ten minutes the number of active fibers in the phallic nerve begins to increase, and a slowly-rising crescendo of activity is observed due to a steady increase in the spike frequency in both newly— and previously— active fibers. Typically this increased efferent discharge gradually becomes patterned into a sequence of impulse bursts. At first the bursts are isolated, but they soon become rhythmic and increase steadily in frequency for the next

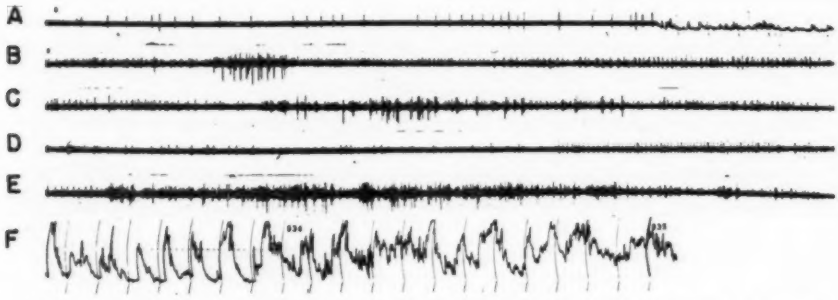

FIGURE 2. A. Oscilloscope record of impulses on the phallic nerve of a normal cockroach, followed by a polygraph record showing the integrated electrical activity from the same nerve. B–E. Oscilloscope records from the same nerve. The roach had been decapitated twenty minutes before the record was made. Note the increased activity. F. Polygraph record of integrated nerve impulses from the phallic nerve of the same roach taken immediately after B–E. The film speed for the oscilloscope record was 25 cm./sec.; the polygraph tape moved 2.5 mm./sec.

thirty minutes (Fig. 2). Individual efferent fibers appear to follow their own burst frequency, giving a rhythmic and often complex pattern to the activity in the compound nerve. In most experiments after thirty minutes the burst pattern usually reaches its full development and remains constant for some hours. Occasional silent periods have been observed, however.

When an active extract of corpora cardiaca is applied to the nerve cord or injected into the head of a roach with an intact nerve cord, the effects of nerve cord transection or decapitation are mimicked (Fig. 3; compare with Fig. 3C, Roeder, Tozian and Weiant, 1960). The sequence of changes in the phallic nerve activity follows the same time course in the gradual development of bursts, and the magnitude of the response is very similar to that released by cord transection. The initial time lag when extract is applied is similar to that seen with decapitation. Desheathing portions of the abdominal ganglia and cord does not decrease this interval. The

only consistent difference is that activity produced by the corpus cardiacum extract is temporary, declining after about one hour. The bursting rhythm becomes irregular and the activity slowly dies down to its original level. If extract is reapplied to the cord every twenty minutes or so, the rhythmic efferent bursts may be maintained for several hours. These effects were observed in about sixty preparations.

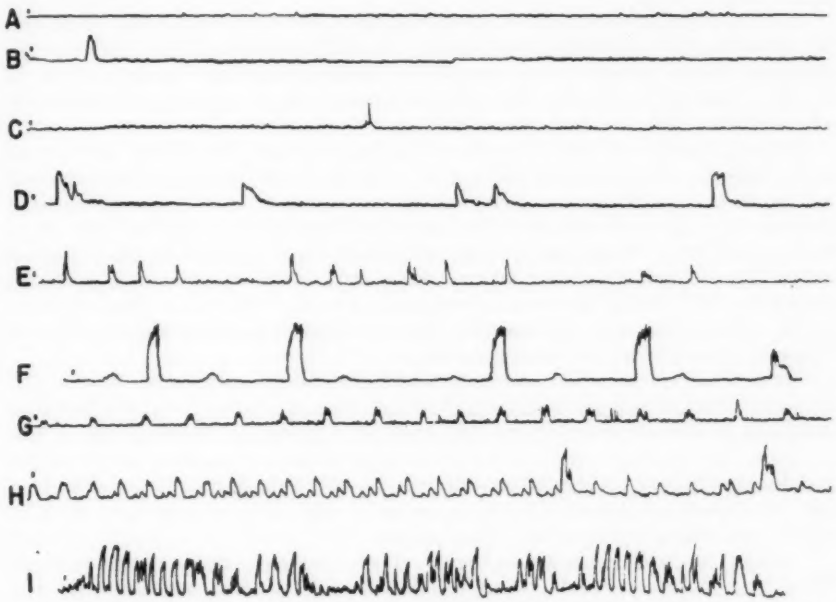

FIGURE 3. Polygraph records showing the integrated phallic nerve activity from the following roaches: A and B. Normal cockroaches. C. Roach with abdominal nerve cord exposed to corpus cardiacum extract, ½ pair/0.01 ml., for 30 minutes (concentration below threshold). D. Roach with abdominal nerve cord exposed for 25 minutes to corpus cardiacum extract, 3 pairs/0.01 ml. from penultimate stage nymphs. E. Roach with abdominal nerve cord exposed to corpus cardiacum extract, 1 pair/0.01 ml., for 30 minutes. F. Roach with abdominal nerve cord exposed to corpus cardiacum extract, 2 pairs/0.01 ml. for seven minutes and (G) for 30 minutes. H. Roach which had had 3 pairs of corpora cardiaca in 0.02 ml. of saline injected into its head capsule 30 minutes before. I. Roach which had had 4 pairs of corpora cardiaca in 0.02 ml. of saline injected into its head capsule 35 minutes before.

The effects of extract concentration. The concentration of corpus cardiacum material necessary to produce the efferent nerve bursts varies both with the size and the species of the donor roaches and with the sensitivity of the test preparation. In general, the larger the adult size of the species, the more active were the extracts of its glands. In terms of their activity when tested on *Periplaneta*, extracts of *Blaberus giganteus* and *Blaberus craniifer* were about equally potent, while the glands of other species ranged down in activity in the following order: *Leucophaea maderae, Periplaneta americana, Byrsotria fumigata,* and *Diploptera punctata.*

The extract is always diluted by the blood of the roach so that quantitative information is difficult to obtain. An extract made from three or four pairs of corpora cardiaca diluted with 0.01 or 0.02 ml. of saline and injected into the head capsule produces pronounced increases in efferent fiber activity in the phallic nerves followed by rhythmic bursts of spikes. Extracts containing one or more pairs of corpora cardiaca per 0.01 ml. of saline when applied to the abdominal cord have similar effects (Fig. 3).

Control extracts were made from abdominal nerve cord tissue and applied in the same manner to the head ganglia and abdominal nerve cords of test roaches. Abdominal cord extract caused occasional transient increases in the activity of both the abdominal cord and the efferent nerves, but the regular and continuing efferent nerve spike bursts were never seen.

In experiments employing more concentrated extracts, containing up to three and a half pairs of corpora cardiaca per 0.01 ml., the initial time lag was unaffected, but the level of phallic motor nerve activity rose more rapidly than with less concentrated extracts and the final frequency attained by the efferent nerve spike bursts was higher. These rapid bursts, when integrated, appeared on the polygraph with a lower amplitude and a shorter duration than the slower-frequency bursts obtained with the less concentrated extracts (Fig. 3, F and G). The burst frequency about thirty-five minutes after the first application of extract provides a rough measure of the potency of the extract.

In four out of seven experiments in which concentrated extracts containing three and one-half pairs of glands per 0.01 ml. were applied to the abdominal cord, the phallic motor nerve activity was blocked after twenty to thirty minutes. This block was only partially reversible by washing. In fact, all attempts to wash out the effects of the corpus cardiacum extracts met with little success if the extract had been on the preparation for fifteen minutes or more. This may have been due in part to the difficulty of irrigating these preparations.

Activity in the abdominal nerve cord. The experiments of Özbas and Hodgson (1958) showed that nerve impulses in isolated roach abdominal nerve cords were blocked when the cords were immersed in extracts of corpora cardiaca. We have confirmed these observations and have also noticed a short period of excitation which seems to precede the block in such isolated cords. It has proved extremely difficult to block nerve impulses in abdominal cords with these extracts if the cords remain *in situ* with the cercal afferents intact and the head and thoracic portions of the cord still attached. When applied to a preparation in which the central nervous system is almost undamaged, the extracts usually cause a brief increase in the level of activity in the abdominal connectives. A tendency for some nerve cells to fire short trains of impulses becomes apparent after the extract has been on the cord twenty to thirty minutes. A period of depressed activity sometimes follows the period of excitation.

Simultaneous recordings made from abdominal connectives and from the phallic nerve do not reveal reciprocal changes in the levels of activity of the cord connectives and the motor nerves after decapitation or after application of extracts containing one to three pairs of corpora cardiaca per 0.01 ml. As the efferent nerve activity increases and bursts of nerve spikes begin to appear on the phallic nerves, the cord activity increases for a time and then usually returns to its previous

level. The cord appears depressed only when concentrations of extracts higher than three pairs per 0.01 ml. were used.

Nerve activity in the thoracic region. The changes induced by corpus cardiacum extracts in the activity of nerve 3 from the metathoracic ganglion and in the thoracic connectives were somewhat more complex than those obtained in the abdominal region. Of twenty-three experiments, fourteen showed increased activity followed by bursts similar to those observed in the phallic nerves. The change occurred twelve to twenty minutes after the extract was applied. In four cases the activity decreased and in four the efferent impulses were blocked. In these cases where the extract caused a temporary or partial block in cord and efferent nerves, twenty minutes were allowed for recovery, but the activity did not return to normal. However, if these roaches were then decapitated, there was an increase in the frequency of the active fibers in both the thoracic cord and nerve 3, and in one case there was also an increase in the number of fibers firing in nerve 3.

In one experiment there appeared to be no change in activity. A temporary decline or cessation of efferent activity was also noted at one period in four of the thirteen preparations which showed an over-all increase in activity and bursts. These changes induced by corpus cardiacum extract correspond closely with those following decapitation or transection of the thoracic connectives (Weiant, 1958).

Changes in activity recorded from electrodes placed on the nerve cord followed very closely the changes in nerve 3 described above. It seems probable that the increased efferent activity was picked up by the cord electrodes, and that this would have masked any decreased activity that might have occurred in the internuncial fibers within the connectives.

In a few cases where the extract reached the exposed thoracic tissues, violent tremors developed in the flight muscles. Since all motor branches from the thoracic ganglia were severed, this suggests that the extract has a direct action on the thoracic muscles.

Abdominal pulsations often coincided with and obscured the thoracic nerve activity. If the abdomen was cut off at this time, there was some decline in the activity in nerve 3. However, this operation rarely brought the activity down to the level recorded previous to the application of extract. If severing the abdomen was followed by decapitation, a slight increase in the frequency of the activity in nerve 3 occurred.

Synaptic conduction. Six experiments were performed using the classic cercal nerve-giant fiber preparation (Roeder, Kennedy and Samson, 1947) to determine the effects of corpus cardiacum extracts on a sensory synapse. Extracts containing one to three pairs of glands per 0.01 ml. were applied or injected and left on the preparation for thirty to forty minutes. The only change which was observed was a slight lowering of the electrical threshold of the synapse in every case.

Comparison with DDT-toxin. Sternberg and Kearns (1952) have reported the presence of an excitor substance liberated from the nervous system of roaches poisoned with DDT. Four attempts have been made to collect this substance using a technique employed by Sternberg. The abdominal cord activity increased greatly after exposure to the saline containing the substance and many impulse trains were seen. No rhythmic spike bursts were observed on the phallic nerves,

however. It was concluded that the DDT-toxin was not the same as the principle in the corpus cardiacum extracts which causes phallic efferent nerve bursts.

Conclusions

The foregoing experiments demonstrate that, except for its reversible action, the extract of corpora cardiaca taken from several species of roaches mimics, in the American roach, removal of the inhibitory system as postulated by Roeder, Tozian and Weiant (1960). The simplest interpretation is that the corpus cardiacum extract suppresses the action of an inhibitory system, there being no evidence that it has any action on excitatory synapses.

If this is the case, a decrease in the activity of those fibers within the nerve cord that mediate the inhibition must occur in the presence of extracts of corpora cardiaca. This could not be detected by electrodes placed in contact with the outside of the nerve cord, although in the abdominal preparations the extract-induced increases in cord activity were transitory and insignificant compared with those occurring in the efferent fibers. Perhaps this is to be expected since the fibers comprising the inhibitory system may occupy only a small portion of the cord substance (Hess, 1958), and from the aspect of an external electrode they are probably shunted by many other fibers either unaffected by, or even released by, the blockage of the inhibitory system.

One perplexing observation in the earlier work (Roeder, Tozian and Weiant, 1960) was that the descending inhibitory system could not be blocked by KCl or by anelectrotonus, the only way of blocking it at that time being by cord transection. To this may be added the current observation that the inhibitory system may be inactivated not only by applying corpus cardiacum extract to its center or point of origin in the subesophageal ganglion, but also anywhere along its path from the head to the locally-originating neurones. It is difficult to visualize a classical nerve pathway operating in this manner, although no alternative mechanism can yet be proposed.

Summary

1. Increased activity and regular bursts of efferent nerve spikes are seen on nerves IX and X from the cercal ganglion of roaches when extracts of corpora cardiaca are applied to the exposed abdominal cord or injected into the head capsule. Such motor nerve activity is usually inhibited in the intact animal by the presence of some influence arising in the subesophageal ganglion (Roeder, Tozian and Weiant, 1960). The corpus cardiacum extracts appear to suppress the action of this inhibitory center. The threshold concentration for this effect is approximately one pair of corpora cardiaca per 0.01 ml. of saline when the extract is applied to the abdominal cord, or three pairs injected into the head in about 0.01 to 0.02 ml. of saline. In decapitated male mantids a similar type of nerve fiber activity is believed to result in behavior associated with mating.

2. The corpus cardiacum extracts seem temporarily to increase the activity of the abdominal nerve cord in preparations with an intact central nervous system. When high concentrations of extract are used, the cord activity is occasionally depressed or blocked. The effects of the extract are only partially reversible by washing the preparation with saline.

3. The activity of certain efferent fibers in the thoracic region is similarly affected by corpus cardiacum extract, the action mimicking that produced by decapitation or cord transection.

4. Corpus cardiacum extracts have no significant effects on the transmission of impulses at the synapse between the cercal nerve and the giant fibers in an electrically stimulated preparation.

5. The DDT-toxin of Sternberg does not have the same effect on the roach nervous system as the active principle in extracts of corpora cardiaca.

6. The corpus cardiacum extracts may be preserved successfully by quick-freezing. Their activity is decreased by prolonged heating, but warming for five minutes at 90° to 100° C. leaves the potency unaffected. Such warming seems to increase by several hours the length of time that an extract will remain potent.

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ON THE HARDENING OF THE CHORION OF THE FISH EGG AFTER FERTILIZATION. III. THE MECHANISM OF CHORION HARDENING IN ORYZIAS LATIPES

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Shortly after fertilization or parthenogenetic activation, soft chorions of fishes are transformed into rigid structures mechanically non-elastic, and chemically resistant. This transformation has attracted much attention particularly in recent years (Nakano, 1956; T. S. Yamamoto, 1957; Ohtsuka, 1957; Zotin, 1958; Rothschild, 1958), and some interesting facts on substances participating in the phenomenon have come to our knowledge. However, the mechanism by which fish chorions are hardened is still a matter of speculation. In an earlier paper of this series (Ohtsuka, 1957), the possibility was suggested that in *Oryzias* egg the process underlying chorion hardening involves an oxidation process. This hypothesis seems to be provided further support in the present paper in which there are presented data on the effects of various chemical agents on the chorion hardening.

The author wishes to express his hearty thanks to Prof. I. Kawakami for his kind direction in the course of this investigation. He is also very grateful to Prof. N. Yoshii for his interest and encouragement.

Material and Methods

The ripe unfertilized eggs of the fresh-water fish, *Oryzias latipes*, were used as material. They were taken from matured females and kept in isotonic Ringer's solution. The overripe eggs of which chorions had slightly separated from the egg surface were discarded. The Ringer's solution used here had the following constitution: $M/7.5$ NaCl 100 parts + $M/7.5$ KCl 2.0 parts + $M/11$ $CaCl_2$ 2.1 parts whose pH was adjusted to 7.3 by adding $NaHCO_3$ (T. Yamamoto, 1944). Sperm suspension was also prepared in Ringer's solution.

Treatments with chemical agents were carried out before or after fertilization at room temperature (22–27.5° C.). In the pretreatment, after some lengths of exposure, aliquots of the eggs were put back into Ringer's solution and then inseminated. Besides, in a few experiments insemination was done in the presence of reagents. The effect was examined with regard to chorion hardening. This is possible to judge indirectly from the degree of chorion elevation; it is generally accepted that the chorion is low when hardening is produced by the reagent whereas it elevates very highly under inhibitory condition. On this account the volume of elevated chorions was calculated 50 minutes after fertilization, according to T. Yamamoto's formula (1940). But the above criterion was found to have its

exceptions as described in the text. Therefore, in all the cases, chorions were torn with glass needles for the determination of their stiffness after volume measurement.

Results

I. Oxidizing agents

The possibility that oxidation takes part in chorion hardening makes it of interest to test whether hardening is caused by means of oxidizing agents. Experiments were conducted on the action of potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate. All the agents were dissolved in Ringer's solution except for periodate. This was made up in Ca-free Ringer's solution to avoid precipitation by calcium.

When unfertilized eggs were subjected to these solutions (pH 7.3) for periods of 15 to 30 minutes, chorion hardening took place in the agents other than chromic acid. Such eggs with hard chorions exhibited no visible cortical changes during each treatment. Here it should be added that some of the eggs were partheno-

Table I

Effect of the presence of oxidizing agents on the chorion changes of cadmium-pretreated eggs at fertilization. Temp. 22.7° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	1.44 ± 0.018	—
Potassium ferricyanide (2×10^{-3} *M*)	0.99 ± 0.015	+
Sodium tetrathionate (5×10^{-3} *M*)	0.99 ± 0.020	+
Iodine in potassium iodide (2.5×10^{-4} *M*)	0.98 ± 0.017	+
Hydrogen peroxide (1.5%)	1.02 ± 0.014	+
Sodium periodate (4×10^{-4} *M*)	0.98 ± 0.012	+
Chromic acid (1×10^{-3} *M*)	1.40 ± 0.016	±
Potassium dichromate (2×10^{-4} *M*)	0.97 ± 0.011	+

genetically activated in both hydrogen peroxide and tetrathionate. In a chromic acid solution the chorion remained unchanged even after more prolonged exposure. It was found, however, that if the solution is acidic, chromic acid, though less effective, is capable of inducing hardening; at pH 6.6 it required a lapse of 60 minutes or more until the chorion became hardened. Then, Ringer's solution of chromic acid with this pH value was employed in the following experiments, but did not give any appreciable effect because of its feeble hardening ability. Treatments with oxidizing agents were carried out soon after fertilization. It was found that except in the case with chromic acid, all the other agents bring about the lowering of chorion elevation as a result of the acceleration of the hardening which follows fertilization.

Table I shows a typical result of experiments in which eggs immersed in a cadmium chloride-Ringer's solution (2.5×10^{-3} *M*) for 10 minutes were transferred to the solutions of oxidizing agents immediately following insemination in Ringer's solution. Cadmium treatment is known to abolish chorion hardening (Ohtsuka, 1957). It will be seen, however, that in these eggs except the chromic acid-treated ones, the chorions elevated were lower so that there developed a narrow perivitelline space.

It is of special interest that chromic acid is less effective than any of the other agents in inducing hardening. The discussion on this point will be presented later.

II. Reducing agents

Experiments were performed using the following reducing agents to determine whether their action on chorion hardening would be reverse to that of oxidizing agents: sodium sulfide, potassium cyanide, sodium thiosulfate, sodium sulfate, sodium thioglycolate, ammonium sulfate and potassium ferrocyanide. These agents were dissolved in appropriately diluted Ringer's solution so as to become osmotically equivalent to $M/7.5$ NaCl, and prepared in concentrations to contain the effective amount of calcium, since the deficiency of the latter blocks hardening of the chorion (Ohtsuka, 1957). The pH of all the solutions was made to 7.3 with HCl or NaOH.

Freshly inseminated eggs were put into each isotonic solution of the reducing agents. Here in both solutions of sulfide and cyanide the chorions were incapable of hardening, but such an effect of the remaining agents was observed only in a

TABLE II

Effect of the presence of reducing agents on the chorion changes at fertilization. Temp. 24.1° C.

Agents	Pretreatment (min.)	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	—	1.05 ± 0.014	+
Sodium sulfide (2×10^{-2} *M*)	—	1.07 ± 0.017	−
Potassium cyanide (3.3×10^{-2} *M*)	—	1.07 ± 0.012	−
Sodium thiosulfate (6.6×10^{-2} *M*)	5	1.06 ± 0.023	−
Sodium sulfate (6.6×10^{-2} *M*)	5	1.07 ± 0.018	−
Sodium thioglycolate (9×10^{-2} *M*)	30	1.06 ± 0.015	−
Ammonium sulfate (6.6×10^{-2} *M*)	5	1.06 ± 0.020	−
Potassium ferrocyanide (4×10^{-2} *M*)	10	1.06 ± 0.013	−

few cases (*e.g.*, in 2 of 9 experiments with thiosulfate). If, however, fertilization was done in the presence of these agents with which the eggs had previously been treated for some periods (5–30 minutes), hardening always failed to occur. Reducing agents were found to be effective in high concentrations. One of the representative results is tabulated in Table II, where it can be seen that under the influence of reducing agents the chorions did not markedly elevate in spite of the inhibition of their hardening. And they were resistant to osmotic pressure of the perivitelline fluid. The factor inhibiting further elevation of the soft chorion in these circumstances is not known. On the other hand, it was impossible to prevent chorion hardening when eggs were inseminated in Ringer's solution after any length of pretreatments. Furthermore, the chorions once hardened could not be converted into a soft condition by treatments with reducing agents.

III. Sulfhydryl compounds

It has not yet been established on the nature of the chorion substrate undergoing hardening in fish eggs. The author (1957) was interested in SH groups,

TABLE III

Effect of 5 minutes' pretreatments with mercaptide-forming agents on the chorion changes at fertilization. Temp. 25.4° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No pretreatment	1.05 ± 0.014	+
Mercuric chloride (2.5×10^{-5} *M*)	1.47 ± 0.022	−
p-Chloromercuribenzoate*	1.48 ± 0.016	−

* Saturated in Ringer's solution.

anticipating that their reactivity might contribute to the chorion hardening. To elucidate this point, two series of experiments were undertaken, one with a mercaptide-forming agent and the other an alkylating agent. Preliminary study by a staining method on the composition of soft chorion seems to demonstrate the presence of SH groups (presumably protein bound-SH groups); no nitroprusside reaction was given, while it was stained with coloured SH reagent (method of Mescon and Flesch), even when this staining was applied after fixing the chorion with 2 per cent trichloroacetic acid.

In the first experiments, unfertilized eggs were placed for 5 minutes in two mercaptide-forming agents in Ringer's solution, mercuric chloride and p-chloro-

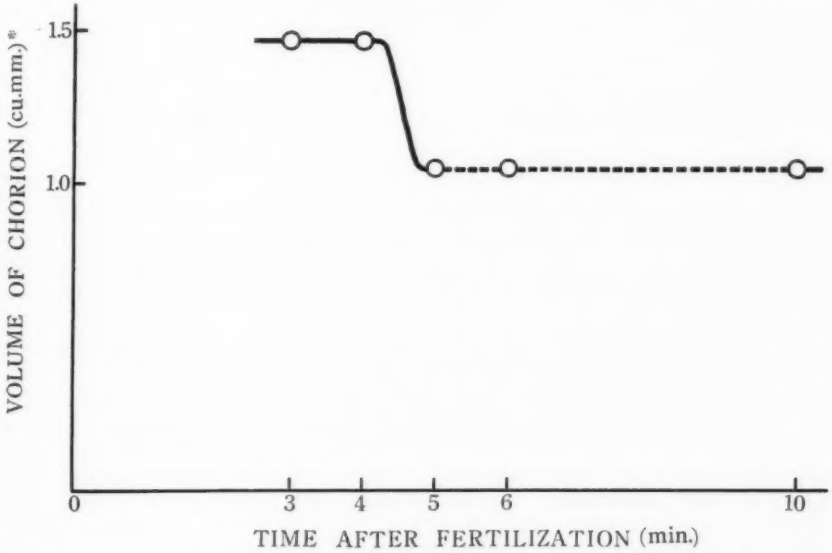

FIGURE 1. Changes in chorion volume in PCMB-Ringer's solution at different times after fertilization. Dotted line shows a period during which the chorion was burst by osmotic pressure of perivitelline fluid.

* Points (5, 10 and 15) of chorion volume in Figure 2 of the previous paper (Ohtsuka, 1957) should be corrected to 0.5, 1.0 and 1.5, respectively.

mercuribenzoate (PCMB). (Lapse of more than 5 minutes in these solutions caused the migration of cortical alveoli towards the animal pole without activation of the egg.) Insemination was carried out in Ringer's solution immediately after the pretreatment. In both cases, chorions were deprived of hardening and there occurred a marked elevation followed by bursting (Table III). This inhibition is due to the blocking of SH groups as shown in the following experiment. According to Anson (1945), the combination of mercury with SH groups can be removed by cyanide. Then, eggs which had been treated with mercaptide-forming agents for 5 minutes as above were consecutively transferred to potassium cyanide-Ringer's solution (3.3×10^{-2} *M*, pH 7.3) prior to insemination. When these eggs were fertilized in Ringer's solution after 5 minutes, reversal of the inhibition was observed. The chorion showed a normal degree of elevation, owing apparently to the occurrence of its hardening.

The failure to harden was also produced by exposing eggs to each mercaptide-forming agent after insemination, and their inhibitory effect could similarly be cancelled upon subsequent treatment with potassium cyanide. Figure 1 represents the results of a typical experiment with PCMB at 25.8° C. If PCMB was applied within 10 minutes after fertilization, further hardening did not proceed

TABLE IV

Effect of the presence of alkylating agents on the chorion changes at fertilization. Temp. 26.2° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	1.05 ± 0.012	+
Iodoacetic acid (3.3×10^{-2} *M*)	1.48 ± 0.017	−
Chloroacetophenone* (0.05%)	1.47 ± 0.024	−

* Saturated in Ringer's solution.

and the elevated chorions retained softness. It should be noted, however, that when this treatment was made about 5 minutes after fertilization or later, such soft chorions became incapable of elevating highly. Unlike the cases of reducing agents described above, they were burst by an osmotic pressure of the perivitelline fluid.

In the next experiments, eggs were subjected to treatment with iodoacetic acid or chloroacetophenone. These alkylating agents were made up in Ringer's solution (pH 7.3). It was found that either short treatment of unfertilized eggs is not inhibitory to chorion hardening when they are subsequently inseminated in Ringer's solution. If the duration of these exposures was long, in the case of chloroacetophenone fertilized eggs underwent cytolysis and the iodoacetic acid-treated eggs lost their fertilizability. When, however, both agents were applied after insemination, the chorions failed to harden. Eggs exposed to alkylating agents soon after fertilization formed considerably high chorions (Table IV).

IV. Urea and lithium bromide

The above experiments clearly indicate that SH groups primarily participate in hardening of the chorion. They might be expected to be available as a possible

donor of hydrogen bonds. If this is really the case, the latter thus given would be responsible for hardening. According to Nakano (1956), the hydrogen bonds are concerned in the mechanical properties of the chorion of *Oryzias*. Attempts were thus made with urea and lithium bromide, which are known to cleave hydrogen bonds. The maximum concentrations at which they were tested were prepared in 3.3×10^{-2} *M* both in Ringer's solution. Eggs were treated before or after fertilization. But neither reagent prevented chorion hardening, even in those cases where unfertilized eggs were exposed for 60 minutes and then inseminated therein. Thus it seems that hydrogen bonds play no role in giving rise to a chorion hardening.

V. Aldehydes

In a staining study, the soft chorion was further found to be periodate-Schiff-positive (Lillie's method), which was blocked by the acetylation technique of McManus. From this result it is evident that a polysaccharide having α-glycol groups impregnates the chorion and produces aldehydes on oxidation (*cf.* Lison, 1953). The question arises whether such aldehydes take part in hardening of

TABLE V

Effect of the presence of aldehydes on the chorion changes of cadmium-pretreated eggs at fertilization. Temp. 27.3° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	1.48 ± 0.017	−
Formaldehyde (0.03%)	0.97 ± 0.014	+
Acetaldehyde (0.5%)	0.98 ± 0.018	+
Acrolein (0.05%)	1.01 ± 0.012	+
Benzaldehyde (0.2%)	0.99 ± 0.015	+

the chorion. To clarify this point, the effect of aldehydes, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde, was investigated. Ringer's solutions of these agents were adjusted to pH 7.3 by adding NaOH.

The results obtained were essentially the same as those with oxidizing agents noted already. The chorions became hardened without egg activation in the presence of the agents other than acrolein. In the latter solution, almost all eggs were parthenogenetically activated 2–3 minutes after their immersion and there took place faster chorion hardening than in the control. Table V illustrates that aldehydes bring about the formation of low chorion when cadmium-pretreated eggs are treated after fertilization in the same way as in the cases of oxidizing agents.

DISCUSSION

As shown in the foregoing pages, the chorion fails to harden under the influence of certain reducing agents. Very high concentrations of the agents are characteristic of this inhibition, suggesting the existence of an antagonistic force responsible for hardening. It has been reported in *Oryzias* egg that a phospholipid in the perivitelline space, which is originated from the cortical layer itself following fertilization or artificial activation, is of prime importance in giving rise to the

chorion hardening, the action of which is regarded as oxidative in nature (Ohtsuka, 1957). Therefore it is most probable that reducing agents inhibit hardening by interfering with this oxidative effect. On the other hand, the oxidizing agents, potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate, were found to have the hardening capacity. The effectiveness of these agents in hardening the chorion is probably due to oxidation. We shall return to this aspect later.

Similar results have been obtained with sea urchin eggs by Motomura and Hiwatashi (1954), who tested the effects of various chemicals on the hardening of the fertilization membrane, and found that the action of reducing agents is opposite to that of oxidizing agents; the former inhibits membrane hardening whereas the latter accelerates it. They offered an explanation that hardening is concerned with polymerization to which both agents are sensitive in a reverse manner. But, as just discussed, the present results can be reasonably interpreted from an oxidative point of view. Still this does not preclude the possibility of polymerization in the hardening of the chorion.

In view of these considerations, it may be allowable to conclude that the oxidation process is involved in the process whereby *Oryzias* chorion is converted into rigid structure. And it appears that it takes part in the initial stage of the hardening process, as is indicated by the fact, for example, that the chorion became hardened even when treatment with sodium periodate was carried out for 10 minutes, during which no changes were visible, and then kept in Ringer's solution. This conclusion is, however, contradicted by Nakano's observation (1956) that colloidal substances, such as gum arabic, gum tragacanth, albumin and gelatin, are capable of hardening *Oryzias* chorion. These substances are unfortunately non-oxidative in chemical nature, their mechanisms remaining obscure.

In a more recent study, Zotin (1958) has found a substance indispensable for the chorion hardening in the perivitelline space of some salmonid eggs. According to him, this substance, which was termed the hardening enzyme, is secreted from the cortical layer, but not from the alveoli present there, upon fertilization or activation, and plays an active role in hardening the chorion. Thus there is a considerable resemblance between phospholipid in *Oryzias* egg and the hardening enzyme of salmonid eggs, particularly with respect to their source and behaviour. Furthermore, Zotin described that hardening of the chorion in salmonids, which is due to polymerization of some substances, is blocked by oxygen deficiency.

It has been shown in the present paper that the SH groups (presumably protein bound-SH groups) of the chorion are essentially necessary for hardening, while their role bears no relation to their possible capacity to give hydrogen bonds. In this connection it should be emphasized that the first three of the effective oxidants noted above, potassium ferricyanide, sodium tetrathionate, and iodine, all oxidize SH groups (Anson, 1945). A similar action, though less reliable, is known about hydrogen peroxide (see Hirai, 1957) capable of hardening as well. Hence these strongly suggest an oxidative conversion of SH into S-S; the latter thus formed may be responsible for chorion hardening. This change must proceed beyond the reversible stage, because a hard chorion is not returned to the soft condition by reducing agents so far as the present study is concerned. Additionally, there is evidence that a chorion polysaccharide having α-glycol groups participates

in hardening. Sodium periodate, another effective oxidant, does change all the available α-glycol groups to aldehydes (*cf.* Lison, 1953). Then, if such a type of oxidation is involved in the chorion hardening, it is worth pointing out that chromic acid likewise produces aldehydes in oxidizing the polysaccharide but it seems without action on α-glycol groups (*cf.* Lison, 1953); this may be the reason why chromic acid is less effective in inducing hardening in spite of its strong oxidative capacity. It is therefore likely that in hardening α-glycol groups are subject to oxidation, resulting in the formation of aldehyde groups. Favoring this idea is the fact that the chorions harden by treatments with aldehyde, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde. Thus it may be said that two substances of the chorion, a protein containing SH groups and a polysaccharide which has α-glycol groups, form a complex undergoing hardening.

From the above account it follows that the reactivities of both sulfhydryl and α-glycol groups contribute to the hardening of the chorion. There must occur some profound changes in these two groups, especially with regard to their, and perhaps reciprocal, oxidative reactions. However, an alternative possibility remains, based on the following facts: According to the present data, SH groups become responsible for chorion hardening about 10 minutes after fertilization. It was further shown that when PCMB, which attacks SH groups, is applied about 5 minutes after fertilization, the chorion retaining softness is incapable of elevating very highly, being burst by osmotic pressure of the perivitelline fluid. These observations reveal some preceding change, probably of polysaccharide, and favor the assumption that oxidation concerns first the α-glycol groups only and then it is followed by that of SH. Significant in this respect is a hardening effect of certain aldehydes mentioned already, and moreover evidence is available that aldehydes are able to combine with SH groups (Schubert, 1936), which latter still remain capable of being oxidized (Anson, 1945).

Thus it may be suggested that hardening is due to the combination of at least two factors in the chorion, an SH group, and an aldehyde produced by oxidation of α-glycol group. And the former would undergo oxidation to S-S. There might be an oxidation polymerization through these disulfide bridges. Very interesting is that the reaction between SH groups and aldehydes is related to a skin tanning (Hirai, 1957). Furthermore, it should be mentioned that potassium dichromate capable of hardening is an excellent tanning agent. Considering these, a tanning reaction appears to take place in the chorion hardening. Rothschild (1958) proposed that fish chorions are hardened by the tanning effect of substance which is discharged from the cortical alveoli at fertilization or parthenogenetic activation. The positive effect of the alveolar substance on chorion hardening has been suggested by Nakano (1956) in *Oryzias* and by T. S. Yamamoto (1957) in *Oncorhynchus*. But the unpublished data on *Oryzias* egg show that hardening is independent of the alveolar substance.

Summary

1. Oxidizing agents, such as potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate, cause hardening of the chorion. Chromic acid is less effective than any of the other agents.

2. In the presence of reducing agents, such as sodium sulfide, potassium cyanide, sodium thiosulfate, sodium sulfate, sodium thioglycolate, ammonium sulfate, and potassium ferrocyanide, the chorions fail to harden.

3. Treatment of unfertilized eggs with mercuric chloride or p-chloromercuribenzoate induces the inhibition of chorion hardening when they are subsequently inseminated in Ringer's solution. The same result is also obtained in the cases where both agents are applied after fertilization. Either inhibitory effect is removed if the treatment is followed by that with potassium cyanide.

4. Iodoacetic acid and chloroacetophenone likewise inhibit hardening when eggs are treated after insemination.

5. Neither urea nor lithium bromide has any effect on the chorion hardening.

6. Aldehydes, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde, are capable of hardening the chorion.

7. Soft chorion is impregnated with a protein containing SH groups, together with polysaccharide which has α-glycol groups.

8. It is concluded that the oxidation process is involved in the mechanism of chorion hardening. It is suggested that hardening is due to combination of SH groups with the aldehydes produced by oxidation of α-glycol groups.

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LOW TEMPERATURE BLOCKAGE OF MOLTING IN UCA PUGNAX

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The fiddler crabs, members of the genus *Uca,* have been the favorite animals for the experimental investigation of molting in brachyurans by American comparative endocrinologists. The facility in collecting and holding these sand and mud flat burrowers and the simplicity of inducing proecdysis by bilateral eyestalk removal have resulted in use of these crabs and this technique by many investigators during the past two decades. Casual note has been made of the variation in proecdysis duration observed, some of which has been attributed to differences in ambient temperatures. Presumably because of limited facilities, no systematic study has been made of the effect of temperature on either proecdysis duration or proecdysis initiation.

In a preliminary observation incidental to a previously reported study of the endocrinological basis of molting (Passano, 1953) a group of 20 male *Uca pugnax* had both eyestalks removed in early winter and were then kept for 35 days in individual bowls at room temperatures diurnally fluctuating between 14° and 21° C. There was a high mortality rate, but of the 8 crabs surviving, none molted. When put in a 27° constant temperature room, three crabs molted after 3, 7, and 8 days. Since eyestalkless *U. pugnax* kept at 23.5° C. showed an average proecdysis period of 19.3 days (Passano, 1953), it appeared as though a 5° reduction in temperature might block or markedly delay a forced ecdysis initiated by eyestalk removal (*i.e.,* by extirpation of the X-organ sinus gland complex).

The experiment reported here has two parts: 1) exposure to a constant test temperature for 23 days, following elimination of the molt-inhibiting hormone (MIH) by bilateral eyestalk removal, and 2) a post-treatment period at the optimum temperature for induced proecdysis, to measure the extent of the previous temperature-induced proecdysis block.

Materials and Methods

Approximately 600 male marsh fiddler crabs, *Uca pugnax* (S. I. Smith), were collected from a salt marsh near New Haven, Connecticut, in late April (water temperature 12° C., air temperature 8°–15° C.) and acclimatized to 15° for one week. Four hundred animals of uniform size were then selected, discarding any whose major chela was missing or undersized, or which had autotomized more than one walking leg, so that the selected crabs had approximately the same blood volume. One eyestalk was removed by cutting across the arthrodial membrane at the base of the eyestalk with fine scissors and each animal was forced to autotomize one of its walking legs, usually the fourth leg on the side opposite to the major chela. The following day the other eyestalk was removed in a like manner. Twelve hours later the surviving eyestalkless animals were divided into ten groups

of 38 crabs each, hereafter called Groups A, B, C . . . J, with the use of a table of random numbers. Subsequent measurement showed that these groups were homogeneous in size distribution with a standard length mean, and standard deviation of the mean, of 18.7 ± 1.2 mm. (Table I). Each animal was put in an individual covered glass dish (or "fingerbowl") with sufficient water (1:1 Long Island Sound Water:tap water; salinity 14‰) to cover the maxillipeds. The water was changed on the sixth, twentieth and thirty-third days after eyestalk removal. Each group was placed in a separate temperature-controlled room (Table I) and kept there for the temperature treatment period of 23 days. Those rooms closest to ambient temperatures, 20° and 22°, showed the largest fluctuations in air temperature, but these rarely exceeded 1° and were rapid and cyclic. A control group of 19 crabs was placed at the warmest temperature after forced

TABLE I

The effect of exposure to different temperatures on ecdysis by eyestalkless Uca

Group	Number (after initial mortality)	Size (standard length, mm.)	Temperature treatment (23 days)			Post-treatment (20 days at 29°)		Total	
			Temp. °C.	% molt	% remaining	% molt	% remaining	% molt	Mean proecdysis duration and S.D.
A	37	18.7	13.1	0	92	46	14	46	37.4± .85
B	37	18.6	15.3	0	86	43	26	43	36.8± .79
C	37	18.5	16.3	0	97	54	19	54	35.7±1.00
D	37	18.6	18.2	0	97	59	16	59	33.4± .97
E	38	18.7	20.0	0	100	58	13	58	31.8±1.05
F	36	18.5	22.2	25	72	31	8	56	24.8±1.15
G	37	18.8	24.4	73	16	11	0	84	19.3± .61
H	35	18.5	26.7	76	17	6	0	81	17.4± .63
I	37	18.5	29.4	89	5	0	0	89	14.8± .73
J	37	18.8	32.2	87	0	0	0	87	14.4± .49
Controls	19	19.0	32.2	0	53	0	11	0	—
	387	18.7±1.2							

autotomy of one walking leg, but as expected, none molted nor showed signs of entering proecdysis. Since the experimental animals were blinded, no regular photoperiods were given. The crabs were not fed, inasmuch as it was impossible to insure equal uptake in the different temperature groups or by individual crabs at the same temperature.

Mortality during the temperature treatment period of the experiment was light. Deaths during the first week (3.2%) are considered to be due to operational mortality and are not included in the tabulated results.

Although some animals successfully completed ecdysis following the experimental treatment, the largest number died during ecdysis or were unable to withdraw all of their appendages. Any crabs dying in Stage E (Drach, 1939) were scored as molted, while those few dying in Stage D_4 were scored as having molted the following day. No animals were kept after ecdysis.

After 23 days, those crabs remaining were examined for evidence of proecdysis initiation. The regeneration of the previously autotomized fourth walking leg was used as an indicator (Bliss, 1956; Jyssum and Passano, 1957). The animals were then slowly equilibrated to 29.4° and kept there for a further 20 days to determine what effect, if any, the initial temperature treatment had had on proecdysis initiation. When the experiment was ended, 43 days after removal of the second eyestalk, 8.5% of the animals remained.

Results

Following elimination of the MIH by bilateral eyestalk removal, a total of 242 of the 368 animals (66%) eventually reached or completed ecdysis. None of the control crabs molted. Table I and Figures 1 and 2 show the differential effect of

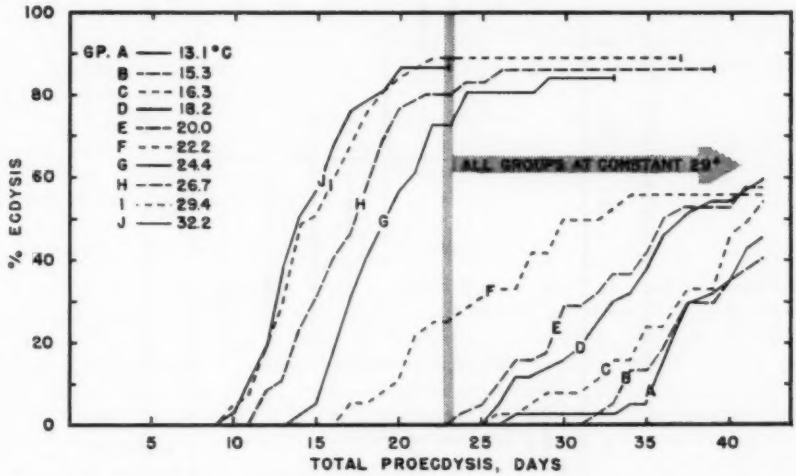

Figure 1. Cumulative percentages of *Uca* molting in each experimental group following eyestalk extirpation.

temperature on average proecdysis duration, the per cent completing proecdysis and the uniformity of response within each group. The low values of the standard errors of the mean for the proecdysis duration of each group are noteworthy.

1. Low temperature treatment, Groups A, B, and C

Exposure to constant low temperature in the range from 13.1° to 15.3° C. blocks proecdysis initiation almost completely, in spite of elimination of the MIH. With a single exception in the group kept at the lowest temperature, none of the Group A or Group B animals initiated discernible proecdysis during the temperature treatment period. Only 14% of these crabs showed basal limb bud (Bliss, 1956) regeneration of their autotomized pereiopod, itself a proecdysis-independent process (Jyssum and Passano, 1957). Again with the single exception already

noted, none reached the ecdysis stage, Stage E (Drach, 1939), in less than 9 days after being placed at 29.4° (Fig. 1). The mean proecdysis duration in the post-treatment warm temperature for Groups A and B combined was 14.1 days with a standard error of the mean of ± 0.48 days. This is not significantly different from the value for Group I crabs which were put directly at 29.4° after eyestalk removal (Fig. 2, shaded bar), although it is shorter.

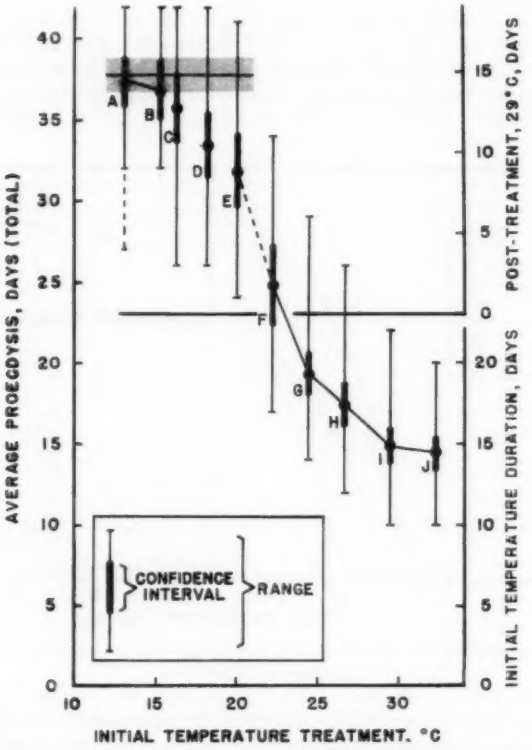

FIGURE 2. The effect of various initial temperature treatments on the proecdysis duration of eyestalkless *Uca*. The shaded horizontal bar represents the confidence interval (13.7–15.9 days) for animals put directly into 29.4° C., transposed to the upper, post-treatment, portion of the graph.

Group A's exceptional animal molted on the fourth day of post-treatment warm temperature. It showed no limb regeneration whatsoever. Since it is probable that this individual had already commenced proecdysis prior to the beginning of the experiment, the increase in the extremes of proecdysis duration caused by this aberrant animal is shown by a dashed line in Figure 2.

Group C, kept at 16.3° for the temperature treatment period, also shows a

post-treatment proecdysis duration, 12.7 ± 1.0 days, insignificantly different from that of Groups A and B. However, half the crabs showed some basal limb bud regeneration after 23 days at 16.3° and one crab, which molted six days later after being raised to 29.4°, had a "premolt limb bud" (Jyssum and Passano, 1957) at that time. The other three animals which molted prior to the tenth post-treatment day again showed no limb regeneration at all.

2. High temperature treatment, Groups G, H, I and J

There was no discernible proecdysis blockage in the groups of animals kept at 24.4° or above (Figs. 1, 2). The two groups kept at the highest temperatures, J (32.2°) and I (29.4°), had average proecdysis durations of 14.4 ± 0.49 days and 14.8 ± 0.73 days, respectively, the slight difference being without statistical validity. The somewhat greater standard deviation of Group I is due to a slight skew of the distribution curve towards the longer proecdysis (Fig. 1). In neither case did any molting occur prior to 10 days, nor 22 days after, removal of the second eyestalk. Molting occurred in 88% of Groups I and J, and of these 94% regenerated the autotomized pereiopod.

Groups H (26.7°) and G (24.4°) also showed a nearly uniform molting response but here proecdysis duration was significantly increased ($P < 0.01\%$; $P < 0.04\%$). As in the groups kept at the highest temperatures, nearly all regenerated their autotomized walking leg.

3. Intermediate temperature, Groups D, E, and F

None of the crabs kept at 18.2° (Group D) or 20.0° (Group E) reached ecdysis during the temperature treatment period, but examination of the regenerating limb buds showed that at least 50% were in proecdysis. During the post-treatment period at 29.4°, approximately half of those crabs which eventually did reach ecdysis had done so before the temperature-blocked animals of Groups A, B and C began to molt (Fig. 1). Nearly all of the Group F molts (22.2°) had molted by this time.

The largest standard deviation from mean proecdysis duration occurred within these intermediate temperature groups, in Group F. Many of those animals in which proecdysis appeared to be blocked failed to survive the 20-day post-treatment period at 29.5° (Table I).

Discussion and Conclusions

Molting after bilateral eyestalk extirpation is due to elimination of molt-inhibiting hormone (MIH) of the medulla terminalis ganglionic X-organ (Bliss, 1953; Passano, 1953) and consequent formation and release of molting hormone (MH) by the Y-organ (Echalier, 1959). MH then causes the sequence of morphogenic and biochemical events collectively termed proecdysis, ecdysis and postecdysis.

The results reported here show that proecdysis duration is strongly temperature-dependent. In *Uca pugnax* a temperature between 29° and 32° gives the shortest average proecdysis duration. Temperatures above this may be deleterious to this

crab, since most of the normal control animals failed to survive the 42 days of this experiment, but it is equally plausible that these animals starved to death. Temperatures somewhat below 29° still permit molting but the proecdysis period is progressively lengthened, doubling in duration when the temperature falls from 30° to 20°.

Temperatures of 15° or below effectively block proecdysis. A comparison between either Group A or Group B and Group I shows that mean proecdysis duration at 29° is nearly the same whether or not the eyestalkless animals had first been kept at 15° for three weeks (Fig. 2). No appreciable proecdysis occurs during this period in these groups. The low temperature treatment did reduce the total percentage molting to one-half its original value (Table I). This is presumably due to the continuous depletion of organic reserves during the three weeks of low temperature treatment starvation. If the experimental animals had been fed, this decrease in the percentage of the group reaching ecdysis would probably have not occurred.

At some temperature between 15° and 22° proecdysis may either fail to commence, or else may be initiated but proceed slowly, after eyestalk removal. The lengthened mean proecdysis durations of the intermediate temperature groups, D (18°) and E (20°), are due in part to proecdysis blockage in some of the crabs. After three weeks at the treatment temperatures, 42% of the 18° animals and 39% of the 20° animals showed no signs of proecdysis initiation. The remaining crabs had initiated proecdysis but its duration was lengthened by the "low" temperature. It is thus to be expected that these intermediate temperature groups are the most heterogeneous in their response of all the temperature groups tested (Fig. 2).

It is scarcely surprising to find an inverse correlation between temperature and proecdysis duration. It is unusual to find that a temperature of 15° or even higher can block completely an animal's growth, even though this species experiences lower temperatures through much of its life cycle. Since it can survive at these lower temperatures for long periods of time, it seems probable that some key reaction, required in proecdysis initiation, is being blocked. The first half of the proecdysis requires MH, since crabs deprived of their Y-organs are permanently blocked in their intermolt stage (Echalier, 1959). Low temperatures might thus prevent the Y-organs from responding to the elimination of the MIH following eyestalk removal. Alternatively, these glands might form and release into the circulation adequate amounts of MH, but the target tissues might be unable to respond to the MH rise. Yet neither of these explanations accounts for the nearly complete correlation of proecdysis blockage and basal limb bud regeneration blockage found in these experiments. Although the main part of limb regeneration occurs only during proecdysis, so that regenerate size is a good index of proecdysis stage (Bliss, 1956), the initial or basal outgrowth of the regenerating limb is independent of Y-organ MH (Jyssum and Passano, 1957) and, unlike proecdysis initiation, is not photosensitive (Bliss, 1956). Therefore if the temperature-dependent proecdysis blockage found here is blocking Y-organ MH formation or activity, some other temperature-dependent step must be blocking basal limb bud formation in these starved animals.

Although a few of the animals which molted in this experiment failed to show

any limb regeneration, 92% did regenerate the autotomized walking leg. Perhaps temperatures of 15° or below limit some metabolic process common to both proecdysis and basal limb bud formation. One of the initial events in proecdysis is the mobilization of hepatopancreas metabolic reserves (Bliss, 1953; Passano, 1960), noted after eyestalk removal by a rise in oxygen consumption (Edwards, 1950; Bliss, 1953) and a fall in R.Q. (Bliss, 1953). Stored fats are utilized for proecdysis integument growth and mineral mobilization (Renaud, 1949). The basic metabolic requirements of the animal, as well as basal bud regeneration, must require storage depletion as long as animals remain unfed. Perhaps the low temperatures prevent sufficient mobilization of hepatopancreas reserves for basal limb regeneration and proecdysis initiation, as well as basal metabolism. It is possible that such mobilization is normally controlled by an eyestalk hormone in the intact animal at higher temperatures, since eyestalk removal causes a rise in oxygen consumption whether or not the Y-organs are intact (Passano, unpublished). It is not known whether the same rise in O_2 consumption follows bilateral X-organ extirpation in the Y-organless animal; if so, the MIH may be controlling proecdysis initiation by limiting the fat depot mobilization necessary for MH synthesis.

An alternative explanation might be developed from the hypothesis that all crustacean growth processes are controlled in an identical manner (Bliss, 1956). Thus basal limb growth would be under MH control, but this MH would originate in some extra-Y-organ site such as the individual limb bud blastema. It would support this alternative if it were found that Y-organless crabs could be forced into proecdysis by initiating mass basal limb bud regeneration. Multiple regeneration in normal crabs can lead to ecdysis even though environmental conditions are unfavorable (Bliss, 1956), but such treatment might activate their intact Y-organs. If such extra-Y-organ sources of MH occur, then low temperatures could be blocking MH formation or activity, irrespective of the hormone's origin.

Uca (including *U. minax* (Le Conte) and *U. pugilator* (Bosc) in addition to the species used here) occurs on the East Coast of the United States as far north as Cape Cod, but does not commonly occur north of the Cape (Rathbun, 1918). The notable discontinuity at Cape Cod in the littoral fauna is primarily due to the cooler summer water temperatures of the Gulf of Maine, yet the temperature experienced by adult *Uca* must be primarily determined by ambient air temperature rather than water temperatures. Since there is no marked air temperature differential between the littoral areas north and south of Cape Cod, it seems likely that the temperature limit on *Uca* acts on growth of its pelagic larvae rather than on post-larval growth. Perhaps, then, the significance of the proecdysis temperature block found in these experiments is that it reflects an identical limitation of proecdysis initiation occurring in *Uca* larvae, so that larvae hatching in the cooler Gulf of Maine waters are unable to grow because they are unable to molt. Caution must be maintained, however, in attributing species limits to simple temperature effects (Moore, 1958).

Summary

1. A uniform population of male *Uca pugnax* was used in studying the effect of 10 temperatures on proecdysis resulting from eyestalk removal.

2. Proecdysis duration is shortest at 29° to 32° C. and lengthens significantly at lower temperatures.

3. Proecdysis initiation is markedly temperature-sensitive. At 15° or below initiation is completely blocked; at 15° to 20° a substantial proportion of the crabs fail to begin proecdysis.

4. Temperatures which block proecdysis initiation also block basal limb bud regeneration (a molt-independent growth process). It is hypothesized that a metabolic event common to both processes is being blocked.

5. The physiological and ecological significance of the proecdysis temperature block is considered.

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THE DEPOSITION OF SKELETAL STRUCTURES IN THE CRUSTACEA. I. THE HISTOLOGY OF THE GASTROLITH SKELETAL TISSUE COMPLEX AND THE GASTROLITH IN THE CRAYFISH, ORCONECTES (CAMBARUS) VIRILIS HAGEN—DECAPODA[1]

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Little is known regarding the basic mechanisms by which certain cells and tissues participate in synthesis and calcification of organic matrices. The gastrolith discs and the branchial exoskeleton provide particularly useful experimental material in the Crustacea for such studies. Attention, in this paper, will be confined to the histological study of the gastrolith discs of the fresh-water crayfish, *Orconectes virilis* Hagen.

The paired gastrolith discs are composed of the cuticular lining of the stomach, the thickened gastric epidermis, and the underlying sub-epidermal connective tissue. They are located in the anterior lateral walls of the cardiac stomach of the crayfish. Their usefulness as sites of activity for the study of cellular processes involved in the synthesis and calcification of organic matrices is evident when it is realized that the epidermis of these modified portions of the stomach wall becomes competent to synthesize and calcify the gastrolith matrix preceding molt. This activity culminates at the end of the premolt period in the formation of hard calcified disc-shaped gastroliths which lie in a sac or pouch now formed between the epidermis and cuticular lining of the stomach. At the same time gastrolith formation occurs, the epidermis of the exoskeleton is participating in resorption of mineral and organic constituents. At the molt, the fully formed gastroliths are shed with the old stomach lining into the stomach. Following molt, these gastroliths are gradually broken down and resorbed. Some of their mineral constituents are resorbed by the gastrolith epidermis and hepatopancreatic epithelium (Travis, in preparation). These mineral constituents are conveyed by the blood to the epidermis underlying the exoskeleton of other areas and are re-used in synthesis and calcification of their organic matrices.

While the description of the presence of gastroliths before molt and their gradual disappearance following molt has been given by a number of authors (Réaumur, 1712; Chantran, 1874a, 1874b; Braun, 1875; Huxley, 1879; Herrick, 1895; Irvine and Woodhead, 1889; Husson, 1952; and others), only certain aspects of the histology of the gastrolith discs have been described by Braun (1875) for the European crayfish, *Astacus fluviatilis,* and by Herrick (1895) for the American lobster, *Homarus americanus*. The histological changes have neither been described with reference to stages of the molting cycle nor to the synthesis of the non-calcified

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skeletal components of the gastrolith discs, and only with brief reference to the synthesis and calcification of the gastrolith itself. Accordingly, it is the aim of the present paper to deal with histological changes which are associated with the formation of non-calcified skeletal components of the gastrolith discs and those involved in the synthesis and calcification of the gastrolith itself.

Materials and Methods

Animals. Since the duration of each stage of the molting cycle, the length of the intermolt periods, and frequency of molts for each size group have not been established for either field or laboratory *Orconectes virilis,* only males ranging in size from 40–49 mm. carapace length and molting during July and August were used in these studies. All animals were previously collected from the Cambridge Reservoir and maintained in the laboratory in concrete tanks containing rocks and soapstone slabs placed over the bottom to provide secluded areas for crayfish retreat and basking. A gently flowing stream of water, ranging in temperature from 21.8–25.0° C., was maintained at a level of approximately 2.5 inches in the tanks.

Stages of the molting cycle. Stages of the molting cycle were determined according to Drach (1939, 1944). This method is based primarily on morphological features of the exoskeleton, and clearly delimits four major stages—A, B, C, D—each of these being divisible into a number of substages. *Postmolt,* consisting of Stages A, B, early, and middle C, is characterized by progressive hardening of the preexuvial layers; formation, progressive thickening and hardening of the endocuticle; the beginning of feeding; and formation and progressive thickening of the membranous layer. The *intermolt condition* (Stage C_4) is characterized by the completion of all components of the exoskeleton—the epicuticle, exocuticle, endocuticle, and membranous layer. At this stage, one of comparative "rest" or "stability," the completed membranous layer with the adhering epidermis can be stripped from the remaining portion of the exoskeleton. *Premolt,* consisting of early, middle, and late Stage D, is characterized by a series of integumentary transformations which occur preparatory for the ensuing molt. The major portions of the old exoskeleton, both organic matrix and mineral salts, are resorbed; the new preexuvial layers, consisting of the epicuticle and exocuticle, are deposited under the old; the animals cease to feed; and the termination of the period is marked by the molt. Stage D_0 is a new stage, previously referred to by Drach in connection with the shrimp molting cycle (Comments at Harvard, 1957). Stage D_0 in the crayfish is a stage in the molting cycle which cannot be distinguished from Stage C_4 by external features of the exoskeleton. The membranous layer with the adhering epidermis can be stripped from the exoskeleton in both stages but in Stage D_0, gastrolith deposition has begun, the gastroliths in this case being thin plate-like structures.

Histological methods. Three to five animals were killed for each specific stage of the molting cycle. The gastrolith discs were quickly removed, placed on a glass slide and straightened in a drop of fixative before they were transferred to bottles containing larger volumes of fixative. Although the author has used various fixatives for general histological studies of crustacean material (1951, 1955, 1957),

Bouin's fixative containing 1% calcium chloride (anhydrous) was used in these studies and has proved to be the best fixative. Fixation was carried out for at least a 24-hour period, followed by washing in 70% ethyl alcohol for the same period, dehydration in 80%, 95%, 97% ethyl alcohol for 30 minutes each, two changes of 100% ethyl alcohol for 15 minutes each and infiltration according to Peterfi's Celloidin-paraffin Method (Pantin, 1948). Sections were cut at 6 μ, deparaffinized, coated with 0.5% celloidin for one minute, allowed to dry and stained with Mallory's triple stain and Ehrlich's haematoxylin and eosin for general histological studies.

Observations and Discussion

The intermolt condition—Stage C_4

Intermolt is marked not only by the completion of the calcified skeletal components and the membranous layer of the rigid exoskeleton, but by the completion of the non-calcified skeletal components of the gastrolith discs. The epicuticle of

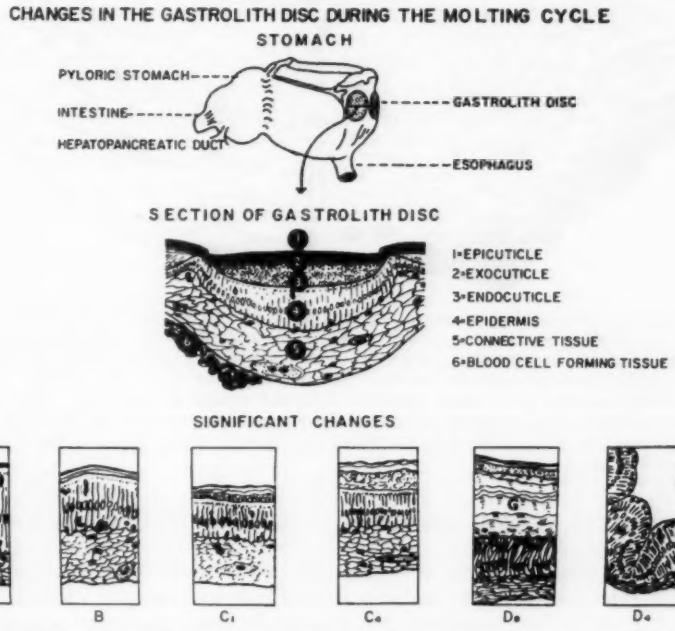

FIGURE 1. Diagram showing the histological changes which occur in the gastrolith disc during significant stages of the molting cycle. Enlarged section of the gastrolith disc is from an intermolt animal (Stage C_4), in which all skeletal components of the disc are completed, more clearly shown in Figure 2. Note significant changes in the development of cuticular components, epidermal height, connective tissue thickness, abundance of reserve cells (represented as black oval bodies). In Stage D_0, note gastrolith (G) forming between epidermis and cuticular components of the disc, and in Stage D_4, note hypertrophy and folding of the epidermis after it has retracted from the fully formed gastrolith and undergone a spurt of growth.

these latter components, adjacent to the lumen of the stomach, constitutes one surface of each gastrolith disc, while the basement membrane of the sub-epidermal connective tissue, adjacent to the hemocoel, constitutes the other surface of each disc (Figs. 1, 2, 4). Although the gastrolith discs, a few millimeters in diameter, are modified portions of the stomach wall, they can be distinguished from the remainder of the stomach wall in Stage C_4 by distinct differences in the structure and composition of their cuticular components and by the nature of their epidermis.

The completed skeletal components of the gastrolith discs of an intermolt animal show three major differentiated layers in contrast to the four observed in

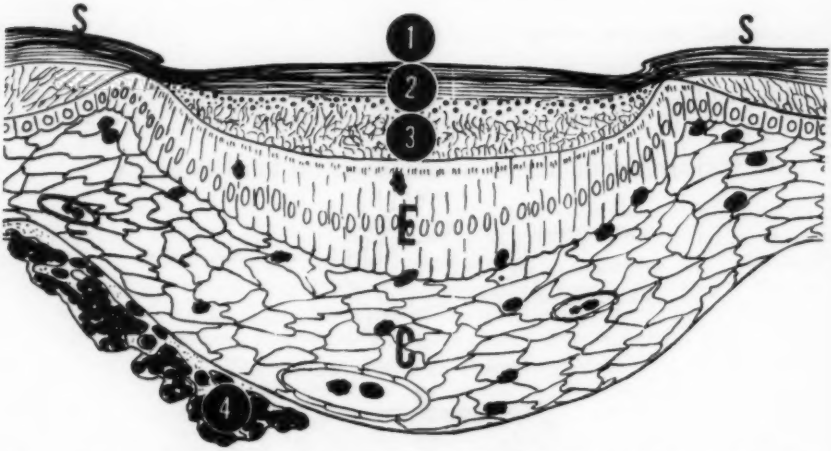

FIGURE 2. Enlarged diagram of the gastrolith disc of an intermolt animal (Stage C_4). Note the three differentiated skeletal layers—(1) epicuticle, (2) laminated exocuticle, (3) endocuticle with characteristic, chemically complex granules which mark the gastrolith disc histologically from the ordinary stomach lining (S). Note the tissue complex of gastrolith disc, which consists of a single layer of columnar epidermal cells (E), the sub-epidermal connective tissue (C), composed of cells of Leydig, and the transient reserve cells (represented as black oval bodies). Also note the blood cell-forming gland (4) and blood vessels in the connective tissue.

the more rigid branchial exoskeleton. Beginning with the lining of the stomach (Figs. 1, 2, 4), these are:

1. *The epicuticle.* This is a very thin non-calcified component, approximately 3–4 μ in thickness and composed of a glyco-lipoprotein complex (Travis, in preparation). It is neither laminated nor crossed by pore canals or tegumental ducts. Elaboration and hardening of the epicuticle begins before molt (Stage D_4) and is completed by Stage C_1. This epicuticle differs from that of the branchial exoskeleton in being thinner and not being calcified or crossed by tegumental ducts. It stains red with Mallory's.

2. *The exocuticle.* This is a laminated component of approximately 8–12 μ in thickness and is also composed of a glyco-lipoprotein complex (Travis, in prepara-

tion). Deposition does not begin until late Stage A and is completed in Stage B. In contrast to the exocuticle of the branchial skeleton the gastrolith disc exocuticle is thinner, non-calcified, lacks pore canals and tegumental ducts, and is formed during a different stage of the molting cycle. The outer zone stains light blue with Mallory's while the inner stains dark blue, with a reddish haze at the junction of the exo- and endocuticle.

3. *The endocuticle.* This is the thickest of the three layers. It reaches a maximum thickness of 15–25 μ in Stage C_4 and stains very light blue with Mallory's. This layer differs from the same layer of the branchial exoskeleton in that it is neither laminated nor crossed by pore canals or tegumental ducts. The endocuticle begins to be deposited in late Stage B and is completed by Stage C_4. It appears as a loose fibrous meshwork and is marked by the presence of round structureless granules (Figs. 1, 2, 4), which also stain red with Mallory's. These granules reach a size of 2.5–3.0 μ, the largest being located immediately under the exocuticle. These are chemically complex granules which contain a glycoprotein complex, a mixed lipid complex, glycogen at certain stages of the molting cycle, and calcium (Travis, in preparation). Such granules are not observed in the endocuticle of the ordinary foregut lining.

The tissue underlying the skeletal components is composed of a single layer of columnar epidermal cells which lie in immediate contact with the endocuticle (Figs. 1, 2, 4). These columnar epidermal cells are approximately 40 μ in height with nuclei of about 5×13 μ. The epidermal layer of the remaining portion of the stomach is a layer of cuboidal or low columnar cells.

The sub-epidermal connective tissue is very similar to that observed in the branchial integument. It is predominantly composed of the loose "spongy" type of connective tissue cells known as "cells of Leydig" (Figs. 1, 2, 4). A number of transient cells, called "protein cells" by Cuénot (1891, 1893), blood corpuscles by Hardy (1892), "reserve cells" (Travis, 1951, 1955, 1957), "lipoprotein cells" (Sewell, 1955), are also evident during the intermolt condition. These transient or reserve cells contain both acidophilic and basophilic granules when stained with haematoxylin and eosin and are usually fuchsinophilic with Mallory's. The granules vary in size, as observed with both the light and electron microscope (Travis and Chapman, II), from less than one micron to around four microns, and have the same histochemical composition as observed in the granules of the endocuticle (Travis, in preparation). The fine-structure studies indicate that the mature reserve cells contain large refractile granules which show variable densities and double membranes, while the immature cells are smaller, may contain clearer non-refractile cytoplasm and smaller granules of variable densities. These cells, among other functions, appear to be intimately involved in transport and release of reserves for synthesis and elaboration of skeletal elements (a more detailed discussion of these cells will be given in a subsequent paper of this series). In addition to these cellular elements, the sub-epidermal connective tissue is well penetrated by branches of the antennary arteries and blood sinuses. Also present in the connective tissue is a conspicuous blood cell-forming gland (Figs. 1, 2), in which stages of amoebocyte or reserve cell development may be seen. This blood cell-forming gland has also been observed by Małaczyńska-Suchcitz and Hryniewiecka (1958). Delimiting the connective tissue from the hemocoel is a basement membrane.

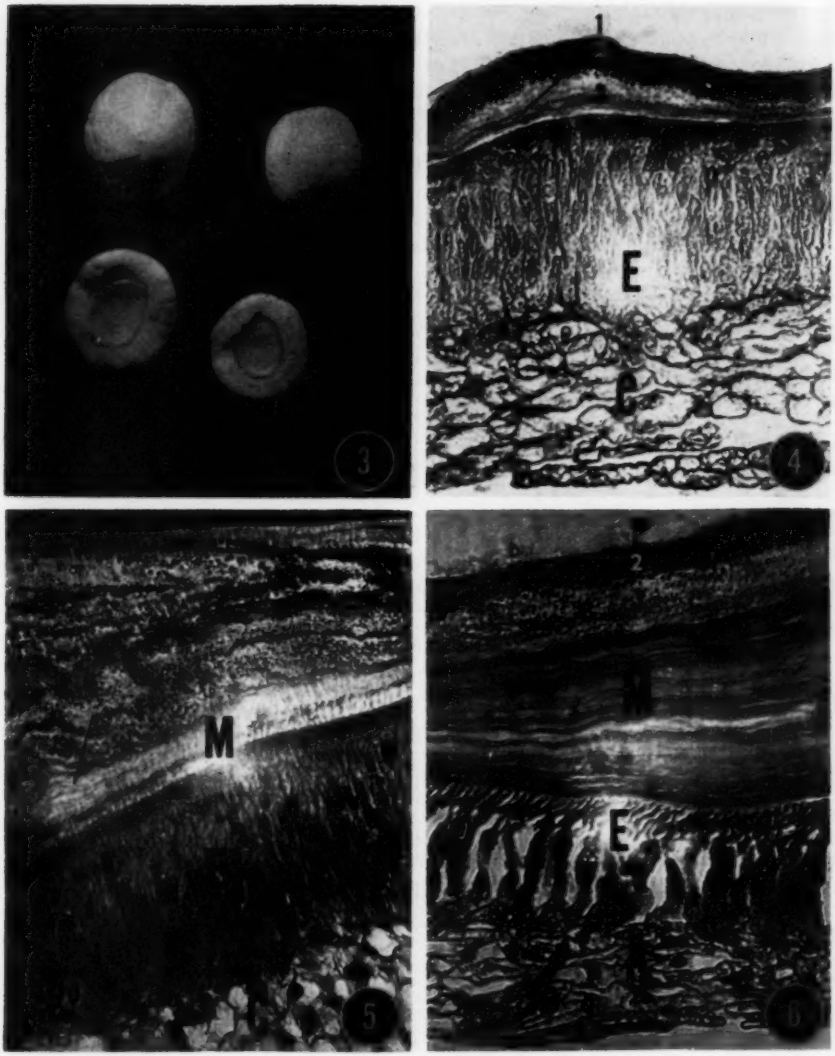

FIGURE 3. Two pairs of gastroliths removed from the gastrolith pouches. The exposed convex surface of the upper pair would lie *in situ* in immediate contact with the epidermis. The concave surface of the lower pair would lie in immediate contact with the stomach lining. 3×.

FIGURE 4. Section of the gastrolith disc skeletal-tissue complex (Stage C_4). The epicuticle (1), the exocuticle (2), the endocuticle (3) containing its characteristic chemically complex granules (arrow), the epidermis (E) and the connective tissue (C). 1800×.

Premolt-Stage D_0 through D_4

During this period of the molting cycle, marked histological changes, associated with gastrolith deposition, occur in the gastrolith disc tissues. In Stage D_0, the epidermis (with cells of about 75 μ in height and bearing nuclei of around 5 × 12 μ) becomes somewhat invaginated. In section, this is evident at the very edge of the gastrolith disc (Fig. 5). These active epidermal cells shortly become branched and attenuated at their apical ends and many intercellular spaces are then apparent between the cells (Figs. 1, 6). Their attenuated apices lie in immediate contact with the forming matrix of the gastrolith. At the ultrastructure level, it is evident that the attenuated apices observed with the light microscope also bear innumerable microvillar equivalents (Travis and Chapman, III). From the microvillar equivalents both organic and mineral material are secreted, some microvillar equivalents secreting granular material into the forming amorphous matrix while others secrete distinctly beaded-fibrous material. The secreted organic components, not observable with the light microscope, undergo polymerization to form the fibrous lamellae (Travis and Chapman, II, III) clearly observed in the matrix of the gastrolith with the light microscope (Figs. 6, 7, 8). Evidence at the ultrastructure level also reveals that calcium transport and deposition appear to occur by two different means (Travis and Chapman, III). The granular and beaded-fibrous components secreted from the microvillar equivalents undergo a change. The former gives rise almost immediately to small crystals of calcite ranging in size from 440–1060 Å, while the beaded components of the latter eventually give rise to the same size crystals, presumably of calcite. Crystal formation from the beaded-fibrous components occurs simultaneously with polymerization changes associated with the formation of the fibrous lamellae of the matrix (Travis and Chapman, III). These small crystals, which form in the matrix of the young gastrolith (Stage D_0) and which are not observed with the light microscope, grow to a size of 7 μ or more in diameter and are clearly observed in ground sections of the completed gastrolith (Figs. 12, 13). These ground sections of the completed gastrolith (Stage D_4) also reveal that there is a shift in the mode of crystal deposition as the youngest and last layers of the fully completed gastrolith are deposited. In this case, crystals are deposited in such a fashion that rows of crystal prisms or rods (Fig. 10) are laid down parallel to the longitudinal axis of the epidermal cells and perpendicular to the parallel lamellae of the gastrolith (Fig. 9). The thickness of the younger layers of the gastrolith is constituted of these crystal prisms or rods, with one crystal stacked vertically above another. This thickness is 490–500 μ as compared to the older portions of the gastrolith (Figs. 9, 11, 12, 13), which constitute a thick-

FIGURE 5. Section through the extreme edge of the developing gastrolith (Stage D_0). Note that the developing matrix (M) is filled with granules similar in appearance and chemical composition to those observed in the completed endocuticle. Also note beginning appearance of fibrous lamellae (arrow). Epidermis is denoted by (E), connective tissue by (C) and reserve cells (R). 1800 ×.

FIGURE 6. Section through a more central portion of the developing gastrolith (Stage D_0). Note the distinct fibrous lamellae of the gastrolith matrix (M) which result from post-secretion polymerization changes, the attenuated apices of the epidermal cells (E) and the intercellular spaces between the cells, and the connective tissue (C). Also note the three differentiated skeletal layers of the gastrolith disc—(1) epicuticle, (2) exocuticle, (3) endocuticle with characteristic granules. 1800 ×.

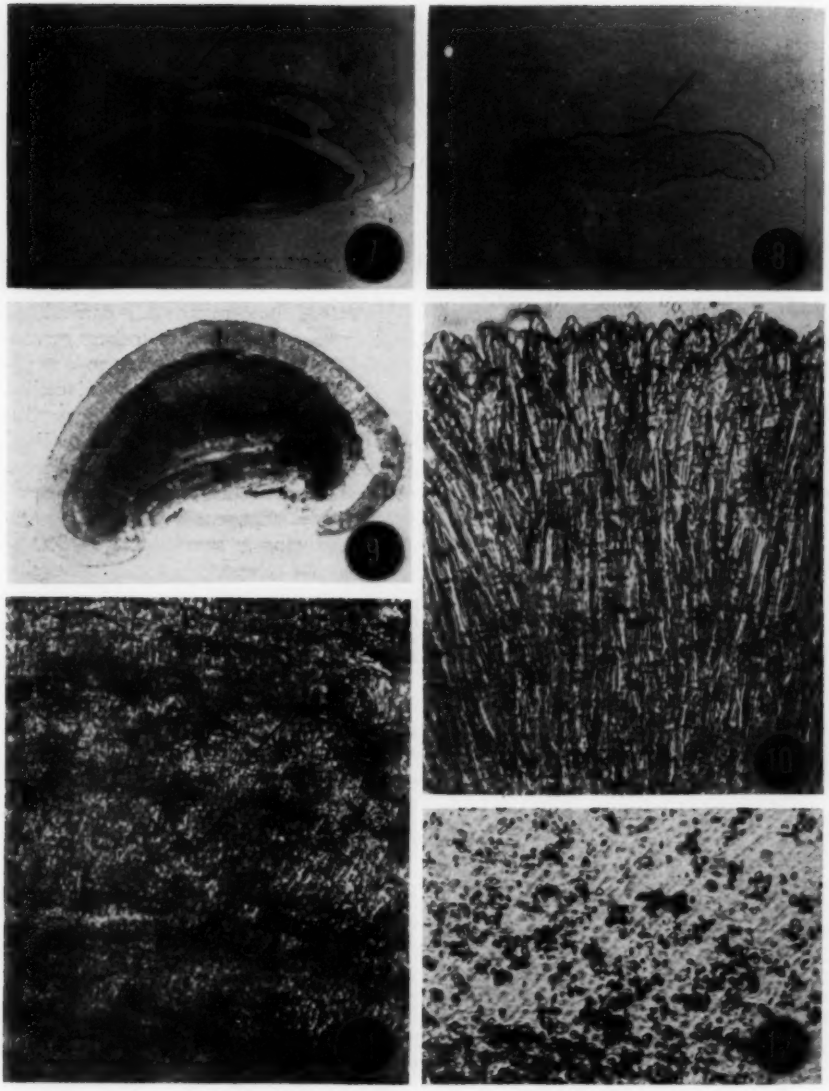

FIGURE 7. A section through the organic matrix of a completed gastrolith (Stage D_4) showing its fibrous lamellate nature. Fixed and decalcified in ordinary Bouin's and stained with toluidine blue. The more convex surface (arrow) would lie *in situ* in immediate contact with the epidermis while the opposite surface would lie in contact with the stomach lining. 16 ×.

FIGURE 8. A similar section through the organic matrix of the early developing gastrolith (Stage D_0), showing its fine fibrous lamellate nature. Arrow indicates surface (younger layers) which would lie *in situ* in contact with the epidermis. 21 ×.

ness of 2500–3000 μ and which show lamellae and crystals oriented at right angles to the prisms. Preliminary x-ray diffraction studies indicate that the main inorganic crystals in both the youngest and oldest layers of the gastrolith are calcium carbonate deposited as calcite.

The sub-epidermal connective tissue becomes greatly compressed or stretched during this stage of the molting cycle. This is largely due to the size of the developing gastrolith which is plate-like in nature (Stage D_0) and has attained a thickness at this time of about 300 μ. By middle Stage D, the gastrolith has increased to a thickness of approximately 1 mm. and by the end of D_4, a thickness of approximately 3–4 mm. is achieved in animals of 40–49 mm. carapace length. Reserve cells remain numerous in the connective tissue and epidermis during the entire premolt period, being heavily concentrated in the apices of the epidermal cells, and achieve their greatest abundance in late Stage D (D_4). During this latter stage the epidermis has completed the deposition of the gastrolith and retracts from it, undergoes rapid growth, accompanied by much folding, and achieves its greatest height (242 μ) and nuclear size of 9×14 μ (Fig. 14). It now begins to elaborate a new epicuticle which is the only skeletal component of the gastrolith disc deposited before molt. In areas of the stomach wall on each side of the gastrolith discs both pre-exuvial layers, epi- and exocuticle, have been deposited. When synthesis of the gastrolith and new epicuticle are achieved by the gastrolith disc epidermis, correlated with the many tasks the epidermis performs in other skeletal areas, molting ensues and the old stomach lining and the completed gastroliths (Fig. 3) are released into the stomach.

Postmolt-Stage A, B and C

Stage A. The tissue complex of the gastrolith disc remains greatly hypertrophied. The epidermis, in contrast to the folded appearance in Stage D_4, is a flat-surfaced tissue, composed of rows of columnar cells, which maintain a height of approximately 182 μ and an average nuclear size the same as that of a D_4 animal (Fig. 15). The reserve cells remain abundant in both the epidermis and connective tissue, with the greatest number being observed in the apices of the epidermal cells. The only skeletal component observed in the gastrolith disc early in Stage A

FIGURE 9. A ground section of a fully developed gastrolith (Stage D_4). The youngest portion (1) would be in contact with the epidermis, and consists of crystal prisms or rods, also indicated in Figure 10, which are laid down parallel to the longitudinal axis of the epidermal cells and perpendicular to the parallel lamellae of the gastrolith. Region (2), also represented in Figure 11, is an older portion of the gastrolith showing the parallel lamellae with crystals oriented at right angles to the prisms of region (1). Region (3) represents the oldest layers of the gastrolith, originally corresponding to Stage D_0, and would lie in contact with the skeletal layers of the gastrolith disc which constitute part of the stomach lining, also represented in Figures 12 and 13. 8.5 ×.

FIGURE 10. Enlarged portion of region (1) from a ground section of a completed gastrolith, depicted in Figure 9. Note crystal prisms or rods of calcite. 400 ×.

FIGURE 11. Enlarged portion of region (2) from a ground section of a completed gastrolith depicted in Figure 9. Note that crystals of calcite in the lamellae (arrows) lie at right angles to the crystal prisms of region (1). 400 ×.

FIGURES 12, 13. Enlarged portions of region (2) from a ground section of a completed gastrolith depicted in Figure 9. Note same arrangement of crystals of calcite—7–70 μ in diameter—as observed in Figure 11. 400 ×.

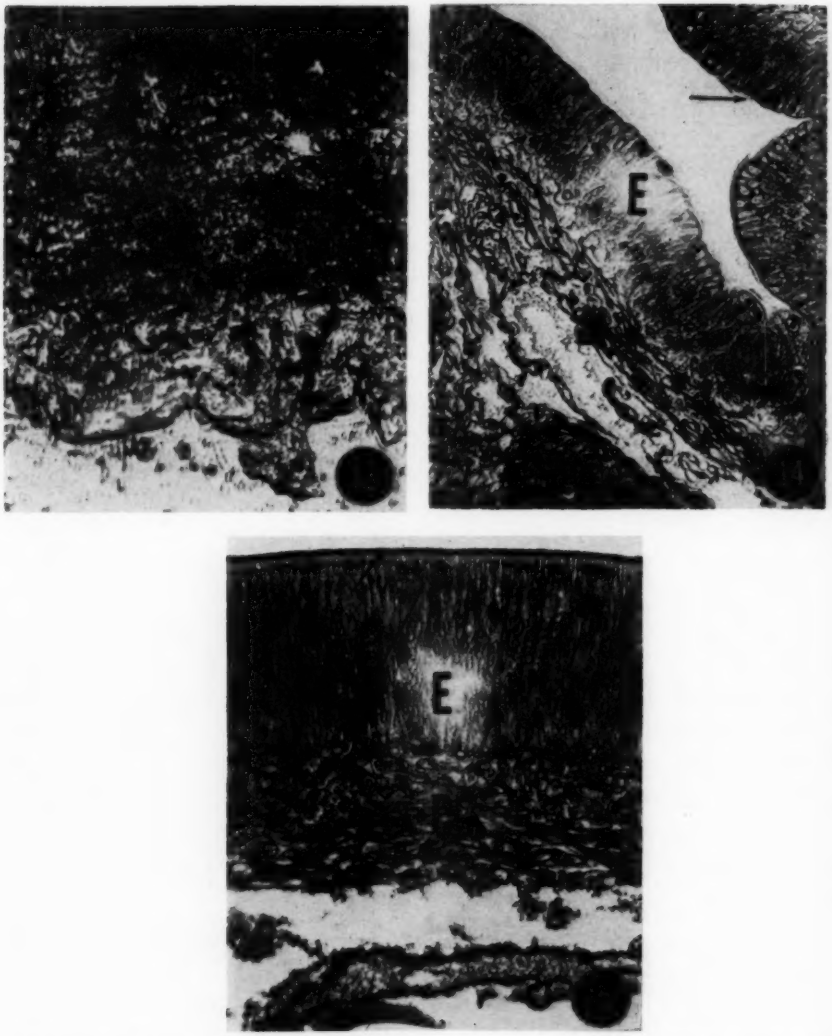

FIGURE 14. Section of gastrolith disc showing greatly hypertrophied and folded epidermis (E), following retraction and growth of this tissue after gastrolith deposition is complete (Stage D_4). Note beginning of new epicuticle formation (arrow) and numerous dark staining reserve cells in the apices of the epidermal cells. 900 ×.

FIGURE 15. Section of gastrolith disc immediately following molt (Stage A). Note the greatly hypertrophied flat-surfaced epidermal cells (E), the epicuticle (arrow), the only skeletal component evident in early Stage A, and the numerous dark staining reserve cells (arrow-R), and connective tissue (C). 900 ×.

is the epicuticle, partly formed and hardened previous to molt and having a thickness of only 1.6–2.0 μ. Curiously enough, the exocuticle does not apparently begin to be deposited until late Stage A and is completed in Stage B, achieving a thickness of approximately 8–12 μ. In the areas of the stomach wall on each side of the gastrolith discs, the exocuticle is completed and endocuticle formation has already begun.

Stage B. This stage of gastrolith disc formation is marked by the completion of the synthesis and elaboration of the exocuticle. It is both fibrous and laminated in nature. This latter aspect is not clearly evident at the microscopic level, but is distinctly apparent in electron micrographs (Travis and Chapman, II). The synthesis and elaboration of the endocuticle also begins during the latter part of this stage but is not completed until late Stage C.

There is a slight decrease in epidermal cell height (112 μ) and nuclear size (5.1 $\times$ 11.5 μ) and an increase in thickness of the connective tissue when compared to the situation observed in Stage A. During this stage the reserve cells are still quite abundant.

Stage C. Early Stage C (C_1) and middle Stage C (C_2) are marked by the reduction in epidermal height (about 80 μ), a slight increase in nuclear size (5 $\times$ 13 μ), an increase in thickness of the sub-epidermal connective tissue, a gradual reduction in the number of reserve cells, and the continued synthesis of the endocuticle, previously described under the intermolt condition. It is during Stage C_1 that the interesting granules, observed in the gastrolith disc endocuticle, become evident at the ultrastructure level, although not becoming distinctly apparent at the light microscope level until Stage C_4. In the tissue complex there is little difference between the early and middle Stage C condition and that observed in the intermolt animal. At the end of Stage C when synthesis of the gastrolith disc skeletal components is complete, the animal has again reached an "intermolt" condition (C_4) from which the entire cycle repeats itself. Further information on the development of the non-calcified skeletal components of the gastrolith disc and of the calcified gastrolith itself will appear in subsequent papers of this series.

Summary

1. The gastrolith discs of the fresh-water crayfish, *Orconectes virilis* Hagen, are located in the anterior lateral walls of the cardiac stomach and are themselves modified portions of the stomach wall.

2. Histologically, the cuticular surface of the completed gastrolith disc (Stage C_4), which forms part of the lining of the stomach, is composed of three differentiated layers: a thin non-calcified epicuticle, formed before molt; a thicker non-calcified exocuticle, laminated in nature, but neither crossed by pore canals, nor tegumental ducts, formed following molt and completed during Stage B; and the endocuticle, the thickest of the three layers, characterized by neither being laminated nor crossed by pore canals and possessing round granules without structure, embedded in a loose fibrous meshwork. The largest of these granules are located in the outer layers of the endocuticle very close to the exocuticle. The tissue complex of the completed gastrolith disc is composed of a single layer of columnar epidermal cells, a loose "spongy" type of sub-epidermal connective tissue, composed of cells of Leydig and a number of the transient reserve cells which are also observed in the

epidermis. The tissue complex is well supplied by branches of the antennary arteries and has a large blood-cell-forming gland.

3. Marked histological changes are observed during Stage D when premolting processes, associated with gastrolith deposition, are set into motion. The epidermis increases greatly in height, invaginates, and begins to elaborate the matrix of the gastrolith which is filled at its lateral edges with granules like those observed in the C_4 endocuticle. At this same time the active epidermal cells take on a branched attenuated appearance at their apical ends, and many intercellular spaces become apparent between the cells. From Stage D_0 through most of Stage D_4 continued synthesis, elaboration, and calcification of the gastrolith matrix occur, its thickness increasing from around 300 μ (D_0) to 3–4 mm. (D_4). Concomitant with these changes is an increase in the number of reserve cells in both epidermis and connective tissue and a stretching, and thus compression, of the sub-epidermal connective tissues, the latter process being correlated with the increasing size of the forming gastrolith. Near the end of Stage D (D_4), the epidermis has completed its task of depositing the gastrolith, retracts from it, undergoes rapid growth accompanied by much folding and achieves its greatest height. It then begins to elaborate the epicuticle, the only pre-exuvial skeletal component deposited in this site. When this task is achieved by the epidermis, correlated with the many tasks it performs in other skeletal areas, molting ensues and the old stomach lining and the formed gastroliths are released into the stomach.

4. Postmolt histological changes in the gastrolith disc are associated with continued synthesis and elaboration of cuticular components. The "intermolt condition" is achieved in Stage C_4, when these synthetic tasks are complete, and from this stage the entire histological cycle repeats itself.

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EFFECT OF TEMPERATURE AND SALINITY ON HEAT TOLERANCE IN TWO GRAPSOID CRABS, HEMIGRAPSUS NUDUS AND HEMIGRAPSUS OREGONENSIS [1]

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Temperature tolerance as an experimental criterion for the demonstration of physiological change has found many uses and has been reported in various ways throughout the literature. Most studies on temperature tolerance have been done on fish, with relatively few experiments on invertebrates. In many cases the lethal point was determined by the temperature at which death occurred when the animal was subjected to slowly increasing temperatures (Huntsman and Sparks, 1924; Gowanloch and Hayes, 1926). As various authors raised the temperature at differing rates, the results could not be compared adequately because the time over which the temperature was increased would be expected to have a marked effect on the final lethal point. Experiments in which animals were maintained at constant temperatures for a period of time provided a more accurate determination of the lethal temperature as well as allowing comparisons among the species. In this type of experiment, either time to death or per cent survival at intervals was noted (Fry, Brett and Clawson, 1942; Brett, 1952; Spoor, 1955).

Acclimation to low and high temperatures has been demonstrated in many animals on the basis of laboratory acclimation, season, microgeography and latitude. In this discussion, "acclimation" includes all types of "demonstrable compensatory change" (Bullock, 1955). Acclimation has been demonstrated by a higher rate function at any intermediate temperature when low temperature acclimated animals are compared with high acclimated ones. Dehnel and Segal (1956) in laboratory studies on the American cockroach, *Periplaneta americana*, found a higher rate of oxygen consumption in equal-weight nymphs and adults after acclimation to 10° C. when compared with 26° C. acclimated animals. Respiration was measured at 20° C. Previously, the culture had been maintained for at least three generations at a constant temperature, 27° C. Edwards and Irving (1943) showed seasonal acclimation in the sand crab, *Emerita talpoida*. At all experimental temperatures below 20° C., oxygen consumption of animals in summer was less than that in winter. Ohsawa and Tsukuda (1956) found seasonal acclimation of response to temperature (exuding of the body) in the periwinkle, *Nodilittorina granularis*. Microgeographical acclimation has been demonstrated in the limpet, *Acmaea limatula*. Samples taken from lower intertidal levels had a higher rate of heart beat than those from higher levels (Segal, 1956).

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Mayer (1914) has shown that at different latitudes there was a similar rate function within a species at the environmental temperatures of the individuals, even though these temperatures were different. The rate of pulsations of the bell of *Aurelia aurita* was similar in animals off Nova Scotia and off Florida with a temperature difference of 15° C. Scholander, Flagg, Walters and Irving (1953) compared many poikilotherms, mostly interspecific but closely related species, from arctic and tropic regions to find the general consistency of acclimation when rates of oxygen consumption were investigated. Dehnel (1955) demonstrated a higher rate of growth in gastropods from high latitudes when compared with low latitude populations.

Another facet of acclimation is its effect on the temperature tolerance of a species. A previous temperature history at the upper levels of the physiological temperature range is known to raise the thermal resistance. Conversely, low temperature acclimation will decrease the tolerance to high temperatures. The rates of gain and loss of heat tolerance appeared to show a consistent trend in diverse groups of animals. In all cases, gain of heat tolerance was much more rapid than its loss. Brett (1946) demonstrated that the goldfish, *Carassius auratus*, required a total of thirty days to acclimate to 28° C. when brought from 4° C. in 8° C. steps, but that acclimation occurred at different rates. Twenty days were required from 4° C. to 12° C., but only three days from 20° C. to 28° C. Loss of heat tolerance in the crayfish, *Orconectes rusticus*, was not completed by the end of the sixteenth day when the crayfish were transferred from 22°–23° C. to 4° C. The heat tolerance lost after thirteen days at 4° C. was regained in about 24 hours (Spoor, 1955). Ohsawa (1956a) using the periwinkle *Nodilittorina granularis*, stated (p. 206), "The acclimatization to higher temperatures in the winter snails is established more readily than the acclimatization to lower temperatures in the summer animals."

The effects of salinity changes on temperature tolerance have been studied less extensively. Broekema (1941) demonstrated that the temperature and salinity relations were interdependent in the shrimp *Crangon crangon* when survival in various combinations of the two was tested; a low salinity was endured better when the temperature was high. Wikgren (1953) showed that there was almost a continuous loss of ions from the crayfish, *Potamobius fluviatilis*, as the temperature dropped below 2° C. from about 18° C. when animals were transferred from tap to distilled water. There was apparently more efficient osmoregulation, normal loss of ions, at higher temperatures (above 2°–3° C.). The lobster, *Homarus americanus*, was shown to have a higher lethal point when both salinity and temperature were high during the period of acclimation. A decrease in salinity with the acclimation temperature held constant resulted in a lowering of thermal resistance (McLeese, 1956).

The two species of shore crabs studied in this investigation, *Hemigrapsus nudus* (Dana) and *H. oregonensis* (Dana), are subjected to wide ranges of temperature and salinity conditions in their natural environment. Experimental parameters of temperature and salinity were based on average seasonal conditions, to determine if these seasonal changes, summer and winter, cause a resulting change in the upper temperature tolerance of the two species. Also, animals were acclimated to various combinations of temperature and salinity, to ascertain any resulting changes in

the temperature tolerance within either season. Because of the morphological similarity and like habitat in this area of these two species, it was hoped that physiological differences, in this case resistance to high temperatures, could be demonstrated. This was found to be the case. Additionally, effects of size, crowding, sex and moulting on the tolerance were noted.

Material and Methods

The experimental animals, *H. nudus* and *H. oregonensis*, were collected from Spanish Bank Beach on the south shore of Burrard Inlet. The Fraser River flowing into Georgia Strait near the collecting beach is the main cause of the recorded fluctuations in salinity occurring in this area. The beach has a gradual slope and changes its contour from sand to rocks and again to sand. The lower sandy area is exposed only on the lower tides. The crabs are found in the rocky intermediate area, the main population of *H. nudus* distributed higher than that of *H. oregonensis*. Individuals of both species, however, can be found almost from highest occurrence to lowest.

The salinity is quite uniform throughout the winter, ranging from about 70 per cent to 80 per cent sea water (based on 100 per cent sea water as 31.88‰). In the spring, with the greater influx of fresh water from the Fraser River, the salinity begins to drop and has been recorded as low as 10 per cent in the summer months. At the same time as the salinity is decreasing, temperature increases, and the relatively static summer state results where the average salinity is about 35 per cent. Daily fluctuations in the salinity also occur, as the incoming tide carries a less saline water along the shore in the collecting region. This muddier Fraser River water is distinct from the water of higher salinity by a visual demarcation several hundred yards from shore.

The two species of crabs are found under rocks where a suitable micro-environment is provided by a pool of water or a bed of mussel shells (*Mytilus edulis*). Both species are found also in damp sand under rocks, often partially buried. Both males and females were used in the temperature tolerance experiments when preliminary experiments showed no difference in the resistance of the sexes. Gravid females or individuals missing any appendages were discarded, as were any soft-shell crabs. A weight range was collected, about 0.2-gm. to 6.0-gm. animals, excluding only very large individuals. Crabs were transported to the laboratory in canvas buckets with damp sea-weed. The experiments reported here were conducted from 1957 to 1959, inclusive.

In this investigation, *holding experiments* refer to those in which the temperature tolerance of the crabs is determined without any previous laboratory acclimation. The animals were tested after at least 24 hours in the laboratory, and during that period they were held at temperature and salinity conditions which approximated those in the field. This time period allowed the gut to be cleared partially so that at the high test tolerance temperatures, deposition of faeces and urine did not foul the water. In *acclimation experiments*, on the other hand, animals were held at previously determined acclimation conditions which differed from environmental circumstances in at least one factor. In these experiments crabs were acclimated for at least one week in the laboratory. Preliminary experiments indi-

cated that acclimation was complete in less than seven days; the tolerance of the two species, therefore, was tested from seven to a maximum of twenty-one days in some experiments. Crabs were not fed at any time during the experiments, and were kept in darkness.

If the acclimation temperature differed greatly from the environmental temperature, the crabs were gradually warmed or cooled until the desired temperature was reached, usually a period of two hours. Plastic dishes containing slightly less than 4 liters of water were used for the experimental animals. Holding or acclimation temperatures were either 5° C. or 20° C., ± 1° C., based on average winter and summer temperatures. The salinities were either 75 per cent or 35 per cent sea water, again average seasonal conditions. Hence, four experimental combinations were used. About thirty-five crabs were placed in each container. Animals were changed, usually once per 24 hours, to water of appropriate temperature and salinity over the acclimation or holding period.

Temperature tolerance tests were conducted by floating the plastic containers in water baths in which the experimental temperature did not vary more than ± 0.1° C. The appropriate salinity, corresponding to that used during the acclimation or holding period, was employed for each tolerance test. Five animals per dish (per 4 liters of water) were tested at 1° C. intervals, from 28° C. to 35° C. The five crabs tested at each temperature ranged in weight from small to large. At each temperature both males and females were tested. Crabs were brought to the test temperature from acclimation or holding temperatures over a period of two to four hours at which time the experiment was begun. Preliminary experiments indicated that more time was unnecessary as a more gradual warming period did not alter the results. The experiment was conducted for 24 hours; dead animals were removed at 12 hours and live ones were changed to clean water. All animals were dried with gauze and weighed to the nearest 0.1 gram. Animals were considered dead only when all movement ceased. Using this criterion as a death point, at no time did animals revive when returned to lower temperatures. Immediately prior to death, the sole motion visible was a slight pumping of water through the gill chambers. Crabs which moulted during the 24 hours of the experiment were discarded.

The relationship of weight and time to death was determined by checking at hourly intervals over the 24-hour period and removing and weighing any dead animals. There was a change of water at 12 hours as noted previously. One set of experiments was used to test the effect of different numbers of crabs per dish on the thermal resistance. All experimental conditions were as described in the foregoing except for the varying densities of animals at each test temperature.

Data were plotted on arithmetic paper as it was believed that this type of plot gave the most accurate representation of the temperature tolerance curve. Each point, indicating per cent survival at any given temperature, represented the average of a number of duplications (each five animals per dish) for those particular conditions. In no instance was this average determined from less than six replications resulting in a total of at least thirty animals; usually more were used. The total graph, then, was based on a minimum of one hundred-eighty animals. No attempt was made to derive a formula for the fitting of each curve; the points were merely joined. The 50 per cent survival temperature was based on the

point at which the survival curve crossed the line representing median survival of the animals. In the graph which represented the relationship between size and time to death, the regression line is eye-fitted.

An analysis of variance was done for the complete summer data mainly to determine if a species difference existed. The difference in tolerance between the species was less during the summer months, so that a significant difference here definitely indicated one for the complete data, both summer and winter. For individual comparisons such as the difference in tolerance between the species at a given temperature-salinity combination, the "Student's" *t*-distribution was used to compare the 50 per cent survival temperatures. Another method was the comparison of the total area under the curve for any two graphs, contrasting the total cumulative per cent survival. The statistical probabilities resulting from the *t* test using this method were approximately the same as when 50 per cent survival temperatures were compared. Since the latter is a value commonly found in the literature, this was chosen as a basis for comparison rather than the total cumulative survival value. In all cases where a statistically significant difference has been demonstrated using the *t* test, the level of significance is based upon the 0.05 probability point, although the probability in some instances is less than the 0.01 level.

Results

SUMMER

Average conditions of salinity and temperature representing the summer months were 20° C. and 35 per cent sea water. Survival values obtained from summer animals with no laboratory acclimation, holding experiments, were considered as the base line curves. These curves were compared with heat tolerances of animals acclimated to 20° C., 75 per cent sea water; 5° C., 35 per cent sea water; and 5° C., 75 per cent sea water. Temperatures at which 50 per cent survival occurred for 12 and 24 hours were used as the basis for comparisons.

Holding Experiment: 20° C. and 35 per cent sea water

Results of the base line experiments showed that *H. oregonensis* was more resistant than *H. nudus* at both 12 and 24 hours. The temperature at which 50 per cent survival was found for *H. oregonensis* was 33.43° C. for 12 hours and 33.16° C. for 24 hours. For *H. nudus* the values were 32.95° C. for 12 hours and 32.50° C. for 24 hours (Fig. 1).

Acclimation Experiment: 20° C. and 75 per cent sea water

Both species showed a marked increase in tolerance after acclimation to 20° C. and 75 per cent sea water. This combination of temperature and salinity differed from the base line conditions only in the salinity, having been changed from 35 per cent to 75 per cent sea water. *H. oregonensis* again showed more tolerance than *H. nudus* with values of 34.50° C. for 12 hours and 34.38° C. for 24 hours, an increase of 1.07° C. and 1.22° C., respectively, over the base line values. The 50 per cent survival temperatures for *H. nudus* were 33.62° C. and 33.45° C. for

12 and 24 hours, an increase of 0.67° C. and 0.95° C. (Fig. 2). The increase found in both species was significantly different from the base line values, P = .05. It is important at this point to note that acclimation to the above conditions provided the most favourable background for withstanding the high test tolerance temperatures.

Acclimation Experiment: 5° C., 35 per cent sea water

Low temperature and low salinity combined appeared to be slightly less favourable for survival during the acclimation period as the number of deaths in both species was greater than with any of the other combinations, but with fewer deaths among *H. nudus*. Results showed the tolerance to be much the same as the base line values, with no significant difference for 50 per cent survival, 33.28° C. and 33.11° C. for *H. oregonensis* and 33.38° C. and 32.64° C. for *H. nudus* for 12 and 24 hours (Fig. 3).

Acclimation Experiment: 5° C., 75 per cent sea water

Acclimation to these conditions did not result in much change in the tolerance when compared with base line conditions. It is possible that the time allowed for acclimation was not of sufficient duration, since animals collected in the winter, when these conditions were found in the field, did show a lowering of the tolerance. However, no change was detected in the temperature at which 50 per cent survival occurred in the summer animals after seven days' acclimation compared with a total of thirteen days' acclimation. The temperatures for 50 per cent survival for *H. oregonensis* were 33.20° C. and 32.73° C. and for *H. nudus*, 33.05° C. and 32.58° C. for 12 and 24 hours (Fig. 4). These values did not have a significant difference from those found in the base line experiments. When these animals, however, were compared with animals acclimated to 20° C., 75 per cent sea water (Fig. 2), a significant difference in tolerance was found, P = .05. Summer animals, then, when acclimated to both high and low temperatures with high salinity constant, demonstrated a reduction of thermal tolerance with the low temperature acclimation.

WINTER

The winter base line represented the heat tolerance of animals collected during the winter months. These were the winter holding experiments. Also, winter animals were acclimated to the three remaining combinations: 20° C., 35 per cent sea water; 20° C., 75 per cent sea water; and 5° C., 35 per cent sea water.

Holding Experiment: 5° C., 75 per cent sea water

The 50 per cent survival temperatures were 32.59° C. and 32.43° C. for *H. oregonensis* and 32.00° C. and 31.79° C. for *H. nudus* for 12 and 24 hours, respectively (Fig. 4). The difference between these winter values and those obtained for summer animals acclimated to these same conditions (winter) is significant, P = .05.

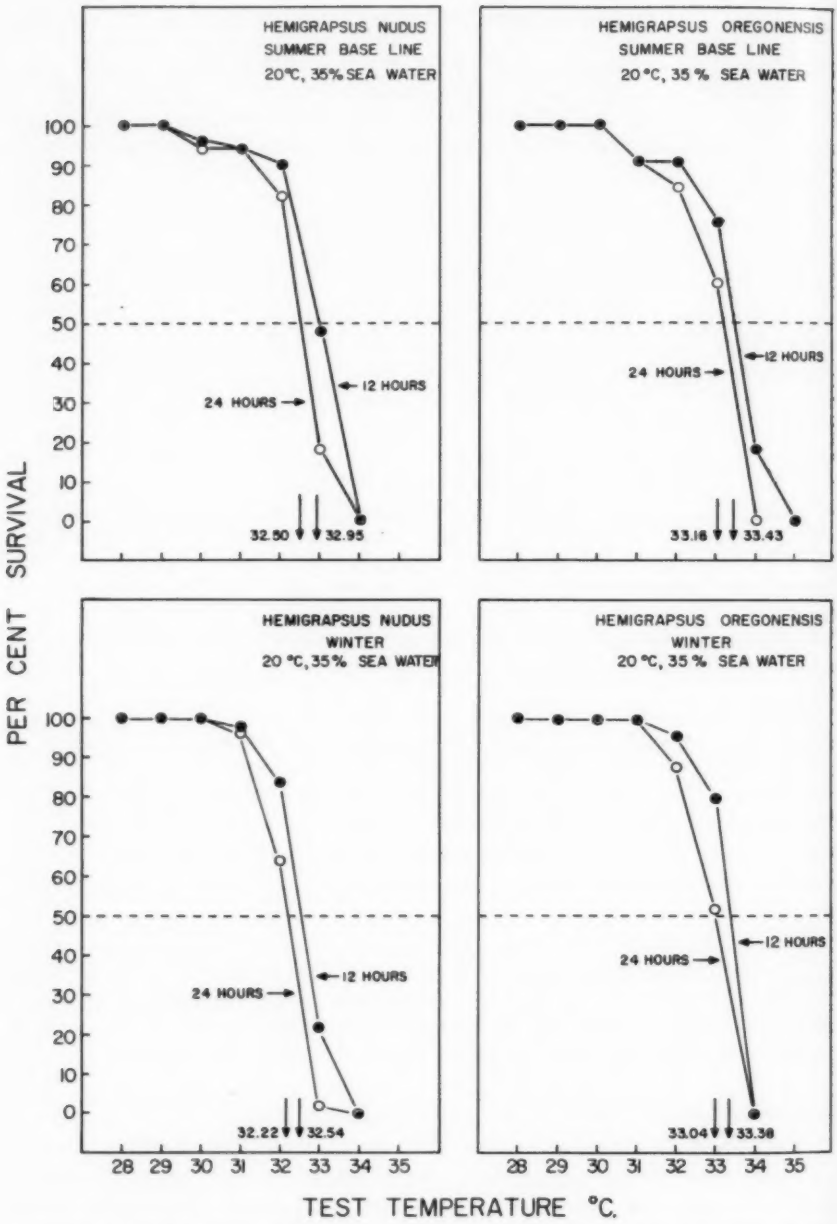

FIGURE 1. The influence of a previous history of 20° C., 35 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

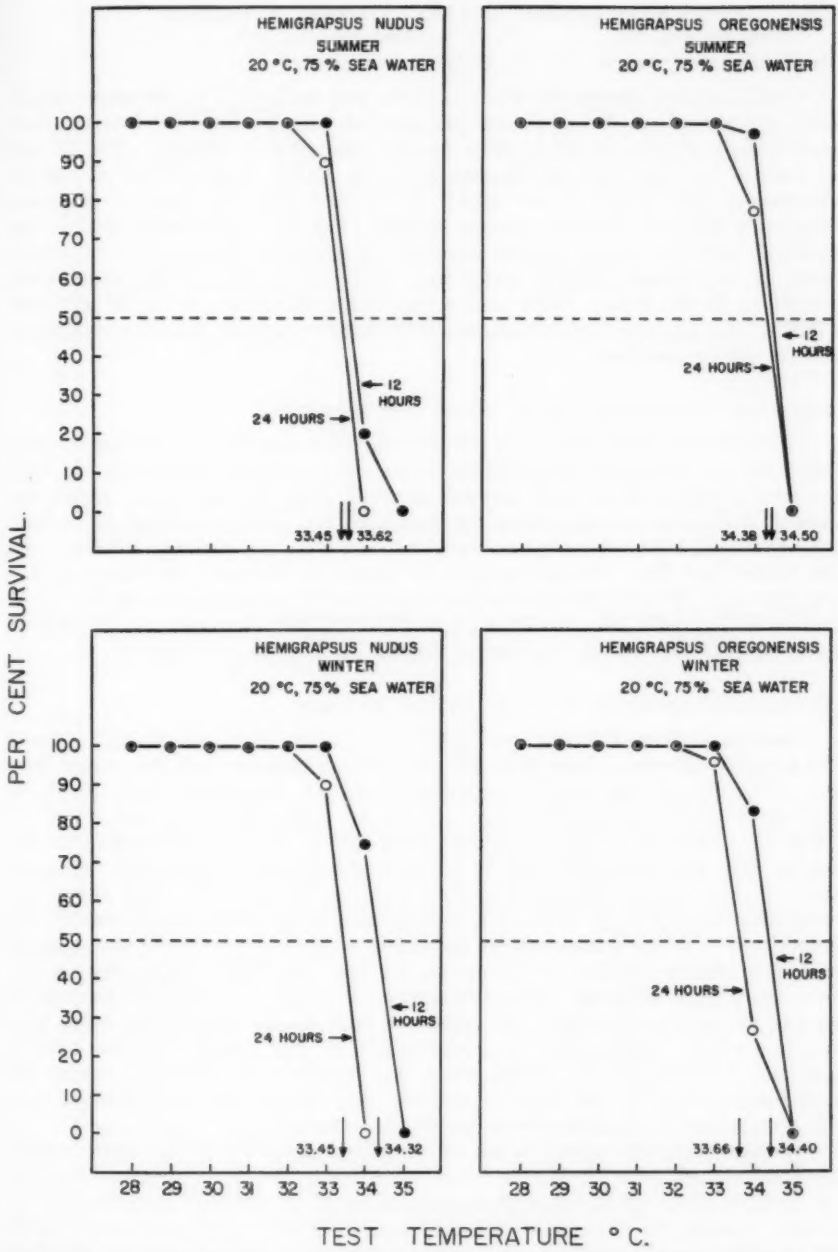

FIGURE 2. The influence of a previous history of 20° C., 75 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

Acclimation Experiment: 20° C., 35 per cent sea water

Crabs collected during the winter months and acclimated to the above conditions, which corresponded to the summer base line combination, were found to have survival values quite similar to those found in the summer months. The 12 and 24 hour 50 per cent survival temperatures were 33.38° C. and 33.04° C. for *H. oregonensis,* and 32.54° C. and 32.22° C. for *H. nudus,* the greatest difference being only 0.41° C. from the summer months (Fig. 1). This meant that in less than one week the winter animals were able to approach the degree of resistance found in the summer animals which may have had several months under these conditions in the field. There was a significant difference in the 50 per cent survival points between winter animals acclimated to summer base line conditions, and the winter base line.

Acclimation Experiment: 20° C., 75 per cent sea water

Acclimation to these conditions provided the most suitable environment to withstand the test tolerance temperatures; there was a marked increase in the temperature at which 50 per cent survival occurred, from the base line. Again, the animals acclimated in the winter approximated the tolerance found in summer animals acclimated to these same conditions, and were significantly different from the winter base line. In one instance, *H. nudus* at 12 hours, the tolerance was even greater. The 50 per cent survival values for *H. oregonensis* were 34.40° C. and 33.66° C., and were 34.32° C. and 33.45° C. for *H. nudus* for 12 and 24 hours (Fig. 2), an average increase of about 2° C. over the base line.

Acclimation Experiment: 5° C., 35 per cent sea water

Animals acclimated to the low salinity, low temperature combination showed the greatest difference, both from the summer counterpart and the winter base line. The 50 per cent survival temperatures for *H. oregonensis* were 30.18° C. and 30.13° C. for 12 and 24 hours, and for *H. nudus* 28.81° C. and 28.68° C. (Fig. 3). This was a drop of approximately 2.5° C. for *H. oregonensis* from the winter base line conditions and 3° C. from the survival temperatures obtained from summer animals acclimated to these same conditions. The drop in the survival temperatures for *H. nudus* was even greater, being about 3.0° C. and 4.5° C., respectively. When winter animals acclimated to both high and low temperatures with low salinity constant are compared, the low temperature acclimation results in a significantly different reduced tolerance ($P = .05$). During the acclimation period, the number of deaths was greater in both species than at any other temperature-salinity combination, a similar result to that found with the summer animals. Contrary to *H. nudus* being the slightly more resistant during the acclimation period in the summer animals, that species was less resistant than *H. oregonensis* in the experiments involving winter animals.

As seen from the results of the above experiments, the 50 per cent survival temperatures for both 12 and 24 hours were higher for *H. oregonensis* than for *H. nudus* in all except one case when the value for *H. oregonensis* was only 0.10° C. lower. Usually the 50 per cent survival temperature for *H. oregonensis* was higher by a much greater amount than this, being in some cases over one degree. Analysis

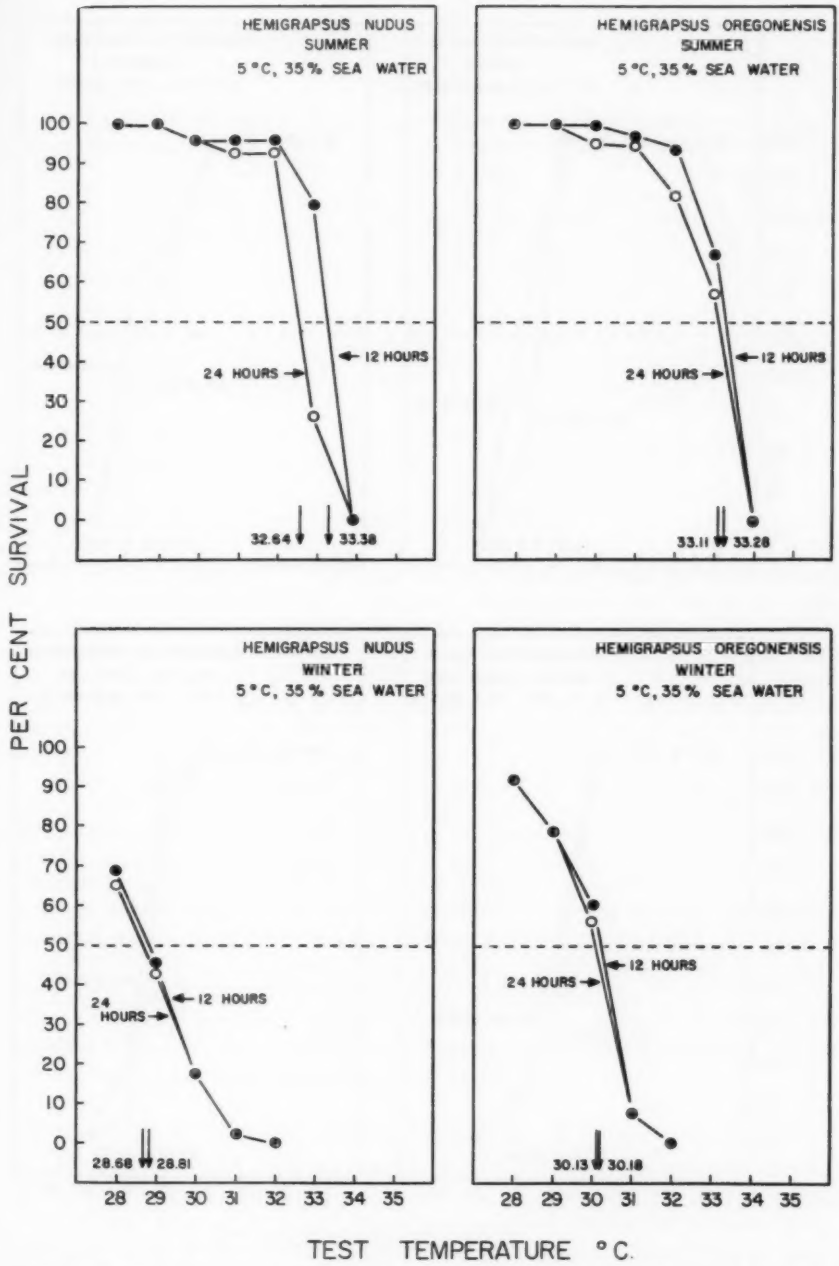

FIGURE 3. The influence of a previous history of 5° C., 35 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

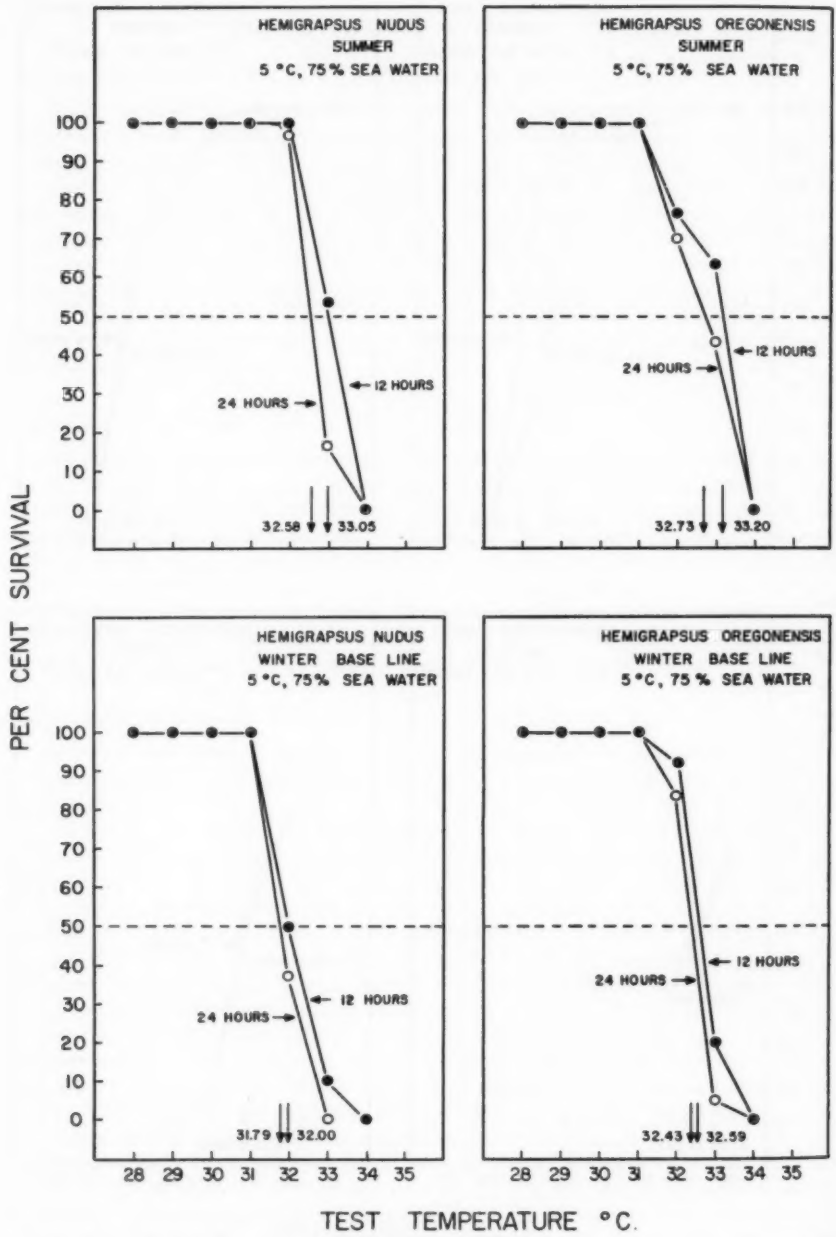

FIGURE 4. The influence of a previous history of 5° C., 75 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

TABLE I

The change in 50 per cent survival temperature with various temperature-salinity combinations

		SUMMER		WINTER	
		H. nudus	*H. oregonensis*	*H. nudus*	*H. oregonensis*
20° C., 35% sea water	12 hr.	32.95° C.*	33.43*	32.54	33.38
	24	32.50*	33.16*	32.22	33.04
20° C., 75% sea water	12 hr.	33.62	34.50	34.32	34.40
	24	33.45	34.38	33.45	33.66
5° C., 35% sea water	12 hr.	33.38	33.28	28.81	30.18
	24	32.64	33.11	28.68	30.13
5° C., 75% sea water	12 hr.	33.05	33.20	32.00*	32.59*
	24	32.58	32.73	31.79*	32.43*

* Base line tolerances in the two seasons, summer and winter.

of variance on summer data indicated that *H. oregonensis* was the more resistant at lethal temperatures, P = .01. The lower resistance is surprising in *H. nudus* as the species is more commonly found at a higher tide level, and it would be expected, *a priori,* that a greater tolerance for high temperatures would be of more advantage to that species. Also, the similarity of the 50 per cent survival temperatures for 12 and 24 hours within each species was consistent throughout. At each test temperature the values were very close, and in many cases were identical. Table I summarizes the results.

Salinity had a pronounced effect on the amount of variability that was obtained in a series of replications of one of the experimental conditions. Whether the acclimation temperature was 20° C. or 5° C., but particularly with the low acclimation temperature, the low salinity (35 per cent sea water) resulted in much more variable survival percentages at the test tolerance temperatures than those found with the higher salinity. Also, the temperature at which 100 per cent survival occurred was a much more tenuous point with the lower salinity. This can be seen readily in any of the low salinity graphs, as there is a gradual slope to the final breaking-off point, whereas with the 75 per cent salinity, the break is abrupt, directly from 100 per cent. It should be noted that the difference in temperatures between the points where 100 per cent or close to 100 per cent survival is found for 24 hours and complete mortality is very slight.

Effects of size, sex, crowding, and moulting

All weights of crabs did not appear to have the same resistance to the high test tolerance temperatures. Smaller animals, in both species, seemed slightly more tolerant than larger ones. There was no direct relationship between weight and time to death, but results indicated that if any animal lived, it was usually less than 1 gram. This seemingly greater tolerance in the smaller crabs was

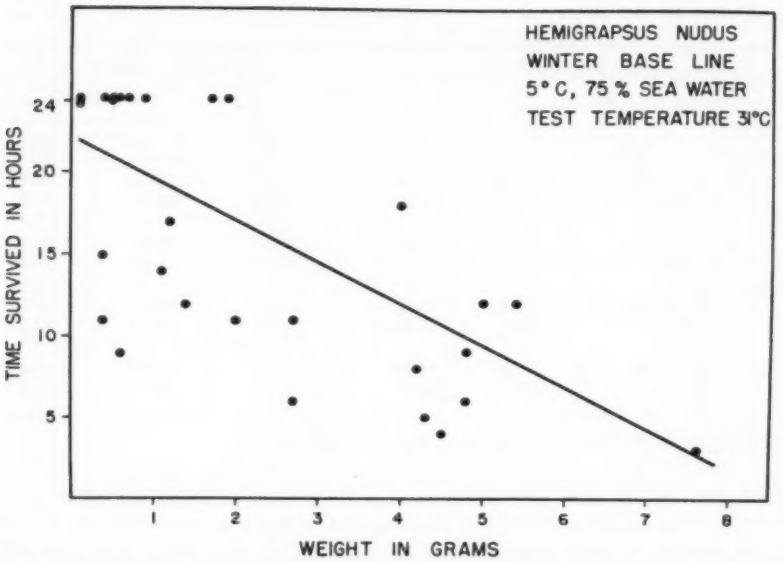

FIGURE 5. The effect of weight on the time survived in hours at 31° C. in *Hemigrapsus nudus*. The animals had a previous history of 5° C., 75 per cent sea water. Each point represents one animal. The regression line is eye-fitted.

prevalent when crabs from all temperature-salinity combinations were tested. One graph was chosen to demonstrate this phenomenon (Fig. 5).

As mentioned previously, both males and females were used in the experiments when the sex was found to have no influence on the resistance to the high temperatures. To the contrary, crowding, that is more than 5 animals per 4 liters of water, caused a marked lowering of resistance to high temperatures. A minimum value of 0.333 liters of sea water per 1-gram live animal weight was found to be satisfactory. When less water was allowed, by including more animals per dish, dying animals apparently affected the water even though they were removed immediately upon death, because those remaining were more apt to die (Fig. 6). Thus, with 5 animals per dish, the 50 per cent survival temperature for 12 hours was 32.95° C., with 10–15 animals per dish, 30.68° C., and with 19–28 animals, 27.86° C. This crowding effect existed only at the high test tolerance temperatures. As was mentioned in the previous section, when there were at least 35 animals per dish at the acclimation temperature of 20° C., no mortality due to crowding was noted.

Since only crabs with a fully hardened carapace were used in the experiments, the effects of moulting on the temperature tolerance were determined from those animals which moulted during the 24-hour course of the experiment. These animals invariably died, even at a temperature where 100 per cent survival normally occurred. A possible change in permeability of the carapace after moulting may have rendered the crabs less resistant to high temperatures.

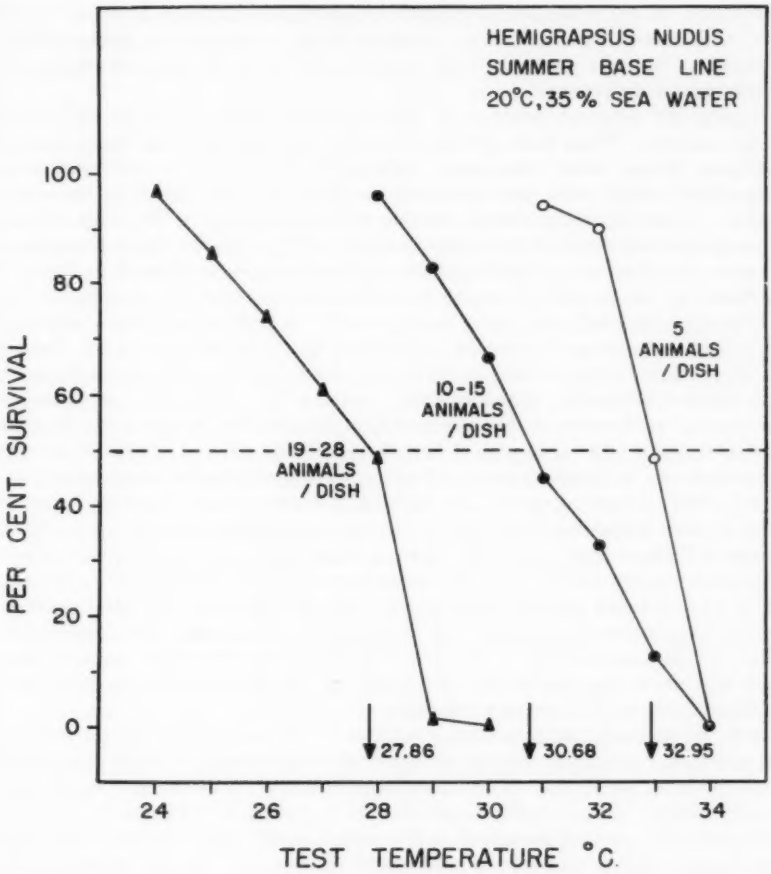

FIGURE 6. The influence in *Hemigrapsus nudus* of differing densities of crabs per 4 liters of sea water on the 50 per cent survival temperature at 12 hours with a previous history of 20° C., 35 per cent sea water: 5 animals per dish (○), 10 to 15 animals per dish (●), 19 to 28 animals per dish (▲). Each point represents the average survival of at least thirty animals.

DISCUSSION

Acclimation to a high temperature, with resulting increase in the high temperature tolerance of the species, has been demonstrated many times (Sumner and Doudoroff, 1938; Brett, 1946; Mellanby, 1954; Spoor, 1955; McLeese, 1956). The two species of crabs, *H. nudus* and *H. oregonensis,* studied here, would seem to be no exception. Temperature tolerance in conjunction with salinity has been studied less extensively, and it was of interest to find that the salinity had a very marked effect on the temperature tolerance of the species. Although results from

the survival curves occasionally differed in summer animals as opposed to winter animals, the trend with regard to tolerance in any particular temperature-salinity combination was the same in both seasons and both species, but there was a definite species difference.

During the summer months, the environmental temperature is high and the salinity is low. When both species of winter animals were acclimated to these conditions, it was found that these acclimated animals had a tolerance at high temperatures which was approximately as great as that found in the summer animals. Likewise, these winter animals when acclimated to the high salinity as well as to the high temperature, demonstrated in both species, again a temperature tolerance very similar to that found in summer animals acclimated to these same conditions, by far the most favourable combination to resist heat death. It is evident that these animals can regain their tolerance to high temperatures very rapidly after a low temperature history by acclimation in the upper part of the physiological temperature range. This rapid gain of heat tolerance has been documented many times. Mellanby (1954) reported that the heat coma point is shifted with experimental acclimation at the upper end of the tolerable range in the mealworm, *Tenebrio molitor* and mosquito, *Aedes aegypti.* The gain in tolerance was rapid, as twenty hours' acclimation produced as much change as longer acclimation periods. Spoor (1955) found complete gain of heat tolerance in the crayfish in less than 24 hours with an approximate 18° C. change in temperature (4° C. to 23° C.). Ohsawa (1956a) determined that gain in heat tolerance by the periwinkle was complete in less than 48 hours when acclimated to 30° C. from 10° C. One exception to a fairly rapid gain in heat tolerance is a recorded time of about twenty-two days for total acclimation in the American lobster when transferred from 14.5° C. to 23° C. (McLeese, 1956). McLeese suggested that the reason for this unusual length of time is that the lobster has a long latent period before onset of demonstrable acclimation, in this case ten days.

A rapid gain of heat tolerance would be of extreme advantage to most intertidal animals. In those animals permanently submerged, a quick augmentation would appear to be of little direct advantage, as bodies of water only slowly change in temperature. For intertidal animals, the increase in temperature may be as much as 10° C. over a period of a few hours in the tidal rhythm. At Spanish Bank, the collecting area for *H. nudus* and *H. oregonensis,* tide pools have been recorded at 26° C. to 28.5° C. In this case, a rapid gain in tolerance, particularly over a period of a few hours, would be most advantageous. Probable increase in salinity, due to evaporation in tide pools during the low tide period in summer months, aids the animal in resisting the high temperatures, as experimental results have indicated that high salinity provides the most favourable environment.

Regarding the low temperature series, the results can not be interpreted as readily. The seasonal difference is marked when compared with the difference found in the high temperature series. The change was consistently to a lower temperature for the 50 per cent survival value in the winter animals. For the winter base line, 5° C., 75 per cent sea water, a drop of about 1° C., from summer animals acclimated to these same conditions, was found in both species at 12 and 24 hours. With the low salinity, low temperature combination, loss in heat tolerance is much greater. In this low temperature series it is clear that the seasonal

alteration in the field environment produces a noticeable reduction of tolerance in the winter animals to high temperatures.

Seasonal change in heat tolerance of a species has been noted by Edwards and Irving (1943) who found a 10° C. difference in the death point of *Emerita talpoida* with summer animals higher than winter ones. Brett (1944) found a change in upper lethal temperatures in Algonquin Park fishes from spring to fall. In most cases, the species studied have been acclimated either solely to various temperatures in the laboratory and the temperature tolerance studied, or the tolerance has been tested at different seasons with no laboratory acclimation. Examples of seasonal comparisons in thermal resistance with the same acclimation temperature in summer and winter are tolerance studies on several species of fresh water fish (Hart, 1952), planaria (Schlieper and Bläsing, 1953, as reported in Fry, 1958) and the rainbow trout (Keiz, 1953). These species were more tolerant of high temperatures in the summer months. Ohsawa (1956a) found that summer periwinkles responded at a higher temperature than winter animals when tested over the same range. Prior to the test, the summer animals had a 48-hour history at 10° C. which corresponded to winter field conditions.

As in all acclimation experiments in this investigation, the summer animals of the low temperature series were kept one week before survival was determined at the test tolerance temperatures. There was no change in the tolerance over the testing interval, seven to thirteen days. This period of approximately two weeks was in agreement with that found necessary by other investigators to demonstrate laboratory acclimation to low temperature. It was evident from experiments on animals collected in winter months that summer animals acclimated to winter field conditions of temperature and salinity in no way approached the reduced tolerance found in winter animals. Likewise, summer animals acclimated to low temperature plus low salinity had a much greater tolerance than was found with the corresponding acclimation condition in winter animals. Insufficient length of time for acclimation is an obvious possibility, but it would be thought that some change would have shown in the tolerance over the period of two weeks. A long latent period before the onset of acclimation to the low temperature is feasible, as a latent period of 10 days was reported in acclimation to a higher temperature in the lobster (McLeese, 1956). Another possibility is that some other factor or a combination of factors influences the acclimation to low temperatures but not to the reverse, as winter animals which were acclimated to summer conditions gave approximately the same results as summer animals. Dehnel (1958) has shown in these two species of crabs, *H. nudus* and *H. oregonensis,* that a simulated seasonal variation of light in the laboratory has a pronounced effect on metabolism measured by oxygen consumption. It is feasible that summer animals would never have the same temperature tolerance as that found in the winter animals solely by acclimating them to winter temperature and salinity conditions. Hoar (1956, p. 365) from experiments on goldfish ". . . concluded that the seasonal variations in thermal tolerance previously noted in fish maintained under constant temperature conditions are photoperiodically controlled. . . ." It is hoped that future experiments will determine this relationship more clearly in these crabs.

Recently, further experiments were carried out on this problem. There was

an attempt to acclimate summer crabs of both species to the low salinity, low temperature combination to determine whether results similar to those found in winter could be obtained. This combination was chosen as acclimation to this temperature-salinity condition resulted in greatly reduced tolerances in winter. The summer animals were acclimated for a total of thirty-one days in some experiments. Resistance appeared to be slightly less when compared with tolerances found after shorter periods of acclimation, but was not as low as those found in winter. Also, experiments were done where the animals had an eight-hour photoperiod corresponding to light conditions in winter with the above temperature-salinity relation. Results again indicated a slightly reduced tolerance but did not appear to differ from those of animals kept in complete darkness. Since two weeks' acclimation in the summer animals did not bring about a change over the testing period, it is important to note that animals cannot be said to acclimate or not acclimate to a parameter merely because there is no evident change.

In nearly all instances low salinity resulted in a lower value for the 50 per cent survival temperature than with high salinity, whether the temperature was 5° C. or 20° C. Gross (1957) gives values for *H. nudus* and *H. oregonensis,* calculated from Jones (1941), which indicate the osmotic gradient maintained by these two species when placed in various concentrations of sea water. The crabs are isotonic to the external environment at 100 per cent sea water (based on 34.6‰). At 75 per cent sea water there is an extremely slight gradient maintained, about 2.5 per cent sea water in *H. oregonensis* and 8.0 per cent in *H. nudus.* The gradient maintained at 35 per cent sea water, however, is about 36 per cent in both species. These values cannot be applied directly to the present experiments, but certainly they can indicate the trend. The maintenance of this gradient presumably results in more work being done by the animal at the lower salinity, and in a greater stress placed on it than at 75 per cent sea water where the gradient is only a few per cent. Additional evidence which suggests more osmotic work is performed at the lower salinity in these crabs is a 22 per cent increase in respiratory rate at 35 per cent over that found at 75 per cent sea water in a 1.0-gram animal measured at 10° C. (Dehnel, 1960). It is probable that the lower 50 per cent survival temperatures with the lower salinity is due to the increased metabolic work necessary at that salinity. The lethal or near lethal temperatures in the test tolerance experiments presumably alone are causing a marked strain on the metabolic activity of the crabs and the additional strain of maintaining a large gradient between the blood and the external medium in the low salinity results in the animal dying at a lower temperature.

In the present investigation, low salinity, combined with low temperature, particularly in the winter animals, was the most disadvantageous acclimation combination for withstanding the test tolerance temperatures. Wikgren (1953) showed the greater loss of ions with temperature decrease when transferred from tap to distilled water. He thought that this greater loss at the lower temperatures could be assigned to depressed absorption of ions and chloride.

A similar relation between salinity and temperature was found by Broekema (1941) in the shrimp, *Crangon crangon.* The salinity optimum for length of life depended on the temperature. With a rise in temperature, the salinity optimum was reached by a downward shift in the salinity. High temperatures generally

were less favourable for survival than low, but low salinity was endured better with a higher temperature. This was similar to the pattern in the two species of crabs studied in these experiments; with low salinity, temperature tolerance was increased when there was a previous high temperature history rather than a low, particularly in winter animals. In addition Broekema showed that the difference between the internal blood concentration and external concentrations in the shrimp was greater at high than at low temperatures and suggested that at low temperatures the limits of life are exceeded sooner than when the temperature is high. Jones (1941) demonstrated a higher osmotic pressure in the blood of *H. nudus* and *H. oregonensis* with a higher temperature, signifying an increase in the degree of regulation. Thus, with a difference in temperature acclimation at the low salinity it is probable that the difference in sustained gradients could carry through to the temperature tolerance tests, and thereby affect the lethal point. Jones, however, showed a great variation in the osmotic pressure of the blood when these crabs were near death from air exposure and felt that this tended to refute a certain lethal osmotic pressure as the primary cause of death.

Broekema's findings, demonstrating that shrimp in a high salinity lived longer at a low temperature, at first appear contrary to the results of this investigation, where the most favourable history for resisting high temperatures was one of both high temperature and high salinity. It must be remembered, however, that the criterion for optimal conditions was quite different, ability to resist lethal temperatures in this investigation, whereas in Broekema's experiments the length of life was the factor. The results cannot be compared directly, but it is felt that they both show the same trend, particularly with respect to low salinities.

Kinne (1958) reported the shifting of heat tolerance in three species of animals, the polychaete *Nereis diversicolor*, the amphipod *Gammarus duebeni*, and the isopod *Sphaeroma hookeri*. The salinity of the pond from which all three species were collected was about 12‰, and in all three species when kept at salinities below this, heat resistance is lowered. Increased heat resistance results in animals from the higher salinities, above 12‰. Kinne suggested that the alteration of water and ion balance resulting in increased water content at extremely low salinities decreases the heat tolerance; the lowered water content at higher salinities favourably affects the resistance to high temperatures. The range of test salinities was beyond the limits of regulation in *Gammarus duebeni* from values given by Verwey (1957) and at the upper salinities in *Nereis diversicolor* (Smith, 1955b). Another explanation than alteration of water content may be necessary when similar changes in tolerance occur over the physiological regulatory range where variation is slight. Since the salinity of the pond where the animals were collected was only 12‰, this would result in a fairly large sustained gradient at least in two of the three species for which data are available. The salinities could cause increased susceptibility to lethal temperatures. The higher salinities presumably within limits would approximate more nearly natural conditions in the body fluids of the animals, to provide a more favourable environment for survival. Another species studied by Kinne (1956), the hydroid *Cordylophora caspia*, has slight osmoregulatory abilities, and again was found to withstand a high temperature better at a high salinity than at lower ones. Possibly the explanation here could be that the higher salinity is nearer the concentration of body fluids of the hydroid,

resulting in a more favourable environment to resist a stress, in this case, high temperature.

The geographic limitation of the polychaete *Nereis* is related to the salinity-temperature balance of its environment. The range of *Nereis diversicolor* in the Baltic Sea apparently is limited by the colder parts of the year when it could not withstand the low salinity expressed as a chlorinity of 4 grams per liter in which it is able to regulate adequately in the warmer summer months (Smith, 1955a). Studies on *Neanthes lighti* indicated that it was able to survive in fresh water in Lake Merced, California, because of its viviparous mode of reproduction where the young are sufficiently developed at birth that they are able to osmoregulate, and that the species is not exposed to severe winter cold. Laboratory experiments showed that the ability to regulate in water of a chlorinity of 1.0 to 0.09 grams per liter was inhibited at 1.5° C. but possible at 13° C. in worms tested in vitro (Smith, 1957).

Animals acclimated to temperatures in the upper part of their physiological temperature range show a remarkable similarity in lethal temperatures. A few comparisons for estimated 12 hours median tolerance among animals acclimated to approximately 20° C. and a favourable salinity, if marine forms, are given in Table II. For the most part the lethal temperature is at least 30° C. In the species difference found in this investigation, the greater thermal resistance of *H. oregonensis* is surprising since it occupies a lower level on the beach. Within the same species, *Littorina litorea*, the individuals collected at high tide level had greater resistance to lethal temperatures than those collected a few yards away at low tide level (Gowanloch and Hayes, 1926). Ohsawa (1956b) determined species difference for the periwinkles *Nodilittorina granularis* and *N. vilis*. The latter occurs in the upper zone of the wide range occupied by *N. granularis*, which was found to be the more tolerant at lethal temperatures, a similar result to that found with *H. nudus* and *H. oregonensis*.

In most animals, then, and possibly all, a previous high temperature history increases heat tolerance and a low one decreases it. Along with this is the influence of salinity where low salinities almost invariably are endured better at a higher temperature, and high temperature, high salinity combined being most favourable for withstanding upper lethal temperatures. Low salinity, low temperature generally is least favourable for length of life and for heat tolerance (Fig. 7).

TABLE II

A comparison of tolerances to high temperature in several species

Species	12 hour median tolerance, ° C.	Source
Hemigrapsus nudus	33.62	—
H. oregonensis	34.50	—
Orconectes rusticus	36.4	Spoor, 1955
O. propinquus	35.0	Bovbjerg, 1952
Cambarus fodiens	35.0	Bovbjerg, 1952
Homarus americanus	28.4–30.5	McLeese, 1956
Hyalella azteca	33.0	Bovee, 1949
Ameiurus nebulosus	33.4	Brett, 1944
Carassius auratus	34.5	Fry, Brett and Clawson, 1942

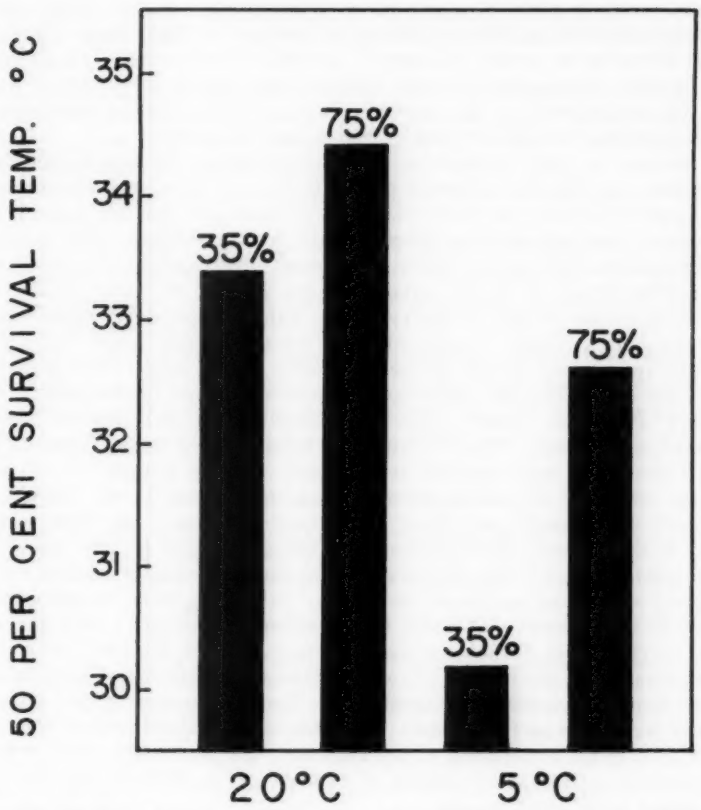

FIGURE 7. Median tolerance temperatures for *Hemigrapsus oregonensis* for 12 hours with previous histories of 20° C. or 5° C. and 35 per cent or 75 per cent sea water. Animals were collected in winter.

The effect of size and sex on heat tolerance was found to vary in different animals reported in the literature. In *H. nudus* and *H. oregonensis*, smaller animals seemed to be slightly more resistant to the high test tolerance temperatures than larger individuals. There was no difference in tolerance between the sexes. Edwards and Irving (1943) found no difference in tolerance in *Emerita talpoida* between males and females, but larger animals seemed slightly more resistant than small ones. In lethal temperature experiments on young speckled trout, *Salvelinus fontinalis*, size did not affect time to death at high temperatures in the same age group (Fry, Hart and Walker, 1946). Hoar (1955) found an increased tolerance at 1°–2° C. in goldfish with an increase in size from 3 to 7.5 cm. In the crayfish, sex and size did not affect heat tolerance (Spoor, 1955). McLeese (1956) concluded that in the size range of lobsters studied, from 21 to 28 cm.,

there is identical response to upper lethal temperatures. Kinne (1958) showed a decreased tolerance in female *Gammarus duebeni* to high temperatures, and increased tolerance in smaller (younger) animals of both sexes. In *Sphaeroma hookeri,* smaller males appeared more resistant than larger ones. There was apparently no sex difference in this species. Thus, it is seen that no conclusions can be drawn regarding the effect of size or sex on heat resistance.

The amount of water available to each animal during the experiments testing heat tolerance was found to influence markedly that tolerance even though animals were removed at death. At least 0.333 liter of water per gm. live animal weight per 12 hours was necessary to prevent death from crowding. The amount of water provided per gm. varies widely in different investigations. Gowanloch and Hayes (1926) tested 10 to 15 periwinkles per 250 cc. of water (0.250 liter), less water than that found necessary in the lethal temperature experiments on these crabs. Brett (1952) tested 40 fish (average weight about 1 gm.) in a lethal bath twenty-two inches square by eleven inches deep. This gave about 2.2 liters per gm. Also the exchange of water was equal to the volume of the tank every 24 hours. Spoor (1955) provided at least 0.1 liter of water for each animal being tested. The size range was from 17 to 42 mm. McLeese (1956) tested 10 lobsters at each constant temperature. Taking a value of 400 gm. as an average weight of the experimental animals, only about 14 cc. (0.0139 liter) at the most was allowed per gram. This was very much less than would be expected if the amount for the decapod Crustacea found in this investigation can be related. A small flow of water into the tank presumably allowed for some exchange of water, but no values are given. Also, the lethal temperatures are low both in comparison with other Crustacea and particularly with the values obtained by Huntsman (1924) for stages IV and V in the lobster. An estimated lethal temperature of about 34° C. is greater than that obtained by McLeese under the most favourable acclimation conditions. Too many animals per volume of water may account in part for results. In many cases in the literature, the volume of water used in the experiment is not indicated, but presumably tests were conducted to ensure a satisfactory density of animals. It is probable that the volume needed varies in different species.

Moulting during the 24 hours of the experiment was found to have an adverse effect on high temperature tolerance in *H. nudus* and *H. oregonensis.* Crayfish moulted successfully in temperatures from 12° C. to 36° C.; the stage in the moult cycle had no effect upon heat tolerance (Spoor, 1955). To the contrary, McLeese (1956) showed that the average survival time for soft-shell lobsters was less than that for hard-shelled individuals. As results from various workers differ, nothing definite can be concluded about a relationship between moulting and death at high lethal temperatures.

Summary

1. The influence on heat tolerance was determined of seasonal change and laboratory acclimation to various temperature-salinity combinations, for two species of grapsoid crabs, *Hemigrapsus nudus* and *H. oregonensis.*

2. There was a seasonal change in 50 per cent survival in both species when base lines from summer and winter were compared.

3. A definite species difference in tolerance to high temperatures was found to exist, but both species reacted similarly to any particular temperature-salinity combination.

4. Acclimation to a high temperature generally increased the resistance to lethal temperatures whereas acclimation to low salinity generally decreased it. High temperature, high salinity was the most favourable combination to withstand the high test tolerance temperatures.

5. Gain in heat tolerance whether the salinity was low or high was rapid, less than one week.

6. Winter tolerances with both low and high salinities in the low temperature series were not demonstrated in the laboratory with summer animals acclimated to these same conditions. Various reasons are suggested which might explain this apparent discrepancy.

7. Moulting during the test tolerance experiments adversely affected the resistance. The number of animals per dish at each test temperature had a pronounced effect on tolerance. The sex of the crabs did not affect the survival, but smaller animals appeared to be slightly more resistant.

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